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THE IDENTIFICATION OF POLLEN FROM SO-CALLED "HAY FEVER" PLANTS

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Pollen grains have been the subject of investigation since the earliest days of microscopical examination of plants. References concerning them may be found as early as the end of the seventeenth century, probably the first reference on this subject being that of Marcello Malpighi (1686) in his famous work 'Anatome Plantarum.' Figure 188, on plate 31, shows drawings of pollen grains of a lily which prove the accuracy of observation of the author. In the accompanying description Malpighi says: "* * * Stamineos loculos, globulorum congerie, quasi atomarum, turgere diximus: Hi diversè configurantur, et colorantur, frequentérque luteum sapiunt colorem, ut in lilio, rosis, et limoniis malis; albescunt verò, diaphaníque ferè sunt in malva, et plantagine. Diversâ pariter donantur figurâ *in lilio croceo, montano albo.* * * *."

From Malpighi up to the beginning of the nineteenth century many references and descriptions of pollen grains are found. Grew pointed out the polymorphism of the pollen grains. Geoffroy spoke of the constancy of size and shape within the species; Needham observed the changes of pollen grains in water prepara-

tion, etc. Very correct drawings of pollen grains appear in Purkinje's (1830) work, where the natural classification of plants is attempted and the pollen grains of each group of plants shown. Von Mohl (1835) gives a very complicated and artificial classification of pollen grains based on different marks of identification. An exhaustive citation of the literature from the time of Malpighi is to be found here. Smith ('76) described and drew the pollen grains of a number of plants, and Anderson-Henry ('76) described the pollen grains of two species of *Fuchsia* in connection with remarks about their hybridization. Edgeworth ('77), Hansgirg ('97), and others made the morphology and anatomy of pollen grains the subject of special investigation, but these authors discussed chiefly European or greenhouse plants which have no direct bearing on the subject in question.

Later there were published a number of papers dealing with the pollen of plants, in which especial attention was paid to so-called "hay fever plants." Among these may be mentioned Scheppegrell's ('22) book, in which he gives a very exact description of the most important hay-fever plants and their pollen. The work is supplemented by many photographs of both the plants and the pollen grains and is certainly of great assistance in the identification of the same. Of some value in identifying pollen grains of hay-fever plants may be mentioned the work of Pope ('25) Koessler and Durham ('26), Waring ('26), Wodehouse ('26), and a report published by the Arlington Chemical Company ('25). Especially in Miss Pope's paper are pollen shapes described, as well as markings, size, color, stickiness, and other characters of importance.

Because of the demand from physicians for a more accurate and definite method than existed for the identification and occurrence of pollen in the vicinity of St. Louis, there was begun in the graduate laboratory at the Missouri Botanical Garden investigations of the pollen of some fifty-five plants regarded as responsible for hay fever. The investigations have been carried on along morphological and microchemical lines for the purpose of devising a key for the identification of pollen grains which occur in the respiratory organs. The results of this work are given herewith in synoptical tables and in a key which it is hoped may

be of some help to botanists and medical men having occasion to identify pollen grains. Attention should be called to the fact that the reactions given may not be typical for pollen grains taken from the respiratory organs or the mucous membrane. It is certain that changes occur in the chemical composition of pollens during their contact with such parts of the body and therefore the chemical reactions noted may be of little or no value under such circumstances. For this reason two different keys have been elaborated; one based on morphological characters and the occurrence of starch, and the other in the form of synoptical tables both morphological and chemical, for the more accurate identification of fresh material.

Methods

The pollen was either taken from mature flowers and tested the same day, or branches with the ripe flower buds were placed in the incubator until the buds opened, when the fresh pollen was examined. All mounts were made in water. After determining the size and shape of the pollen grains they were stained with "Acid Nigrosine" to determine the number of pores and the presence or absence of lids, after which the other chemical tests were applied.

In all cases the reagent was added in small quantities to the water mounts, except where the pollen had to be tested directly in the reagent in question (i. e., Millon's reagent). Here strong chemicals, such as concentrated acids or lyes, were added drop by drop to avoid a too rapid reaction. In every case the reactions were watched for a long time, or repeated. Tests made from dried herbarium material after it had been kept for years showed that it is possible to identify pollen grains morphologically, the size, shape, and even the number of pores being readily determined. It was not, however, found possible to apply chemical tests to dried pollen, since practically none of the reactions occurring on fresh material took place on the dried specimens.

All investigations and microchemical tests were made with a Zeiss microscope, objective "D" and "ocular No. 3."

SHAPE

The most common shapes of the pollen grains investigated were either spherical or elliptical. Tetrahedral and polyhedral grains also occurred, these outlines being modifications of a sphere caused by the pores producing an angular surface. Mounted in water these shapes are especially distinct, due to the difference in swelling of the pollen wall and the pores.

The pollen of *Pinus austriaca*, like that of most of the conifers, is characterized by the presence of two sac-like projections. These air-sacs are apparently nothing more than the enlarged covers of the pores, the grain itself being typically elliptical (see "Acid Nigrosine" below).

SIZE AND COLOR

The prevailing size of the pollen grains examined varied from 15 to 40 μ in diameter. A few were larger, such as *Ailanthus glandulosa*, 20–50 μ, *Pinus austriaca* (without air-sacs), 40–55 μ, *Taraxacum officinale*, 35–50 μ, *Polygonum persicaria*, 48–70 μ. For each species the size of the pollen grains is constant within the limit given.

The prevailing color of the pollen grains investigated was yellow; in a few cases, noted in the tables, a brown, gray-yellow, or greenish yellow color occurred. The color is of no diagnostic significance, however, and no use is made of it in the key.

SURFACE MARKINGS

Surface markings are important in distinguishing pollen grains, especially if the pores or the thinner places in the pollen wall are considered. Even ignoring the pores, the pollen surfaces of the different species show marked differences. While many are smooth, others have a spiculated or warty surface with spines of different kinds and shapes, with irregular protuberances, reticulations, oil drops, etc.

When the pores are not distinctly visible, they can be brought out more distinctly by certain acids. Reference to such reactions will be found in the synoptical tables. In general, two kinds of pores may be distinguished, large ones and very fine ones, but occasionally there may be a combination of both sizes, so that

the pollen may be classified under one of three heads. The large pores may be covered with a distinct lid or closed only with a very delicate membrane, whereas the fine pores are closed with the delicate membrane only. Consequently, according to the nature of the pore covering, a different effect is obtained by treating with certain chemicals or stains. Many of the well-known stains were tried, but no single one was satisfactory for the different pollens. Therefore there was devised a combination of acetic acid and nigrosine, described later under the heading "Acid-Nigrosine," which gave satisfactory results in all cases.

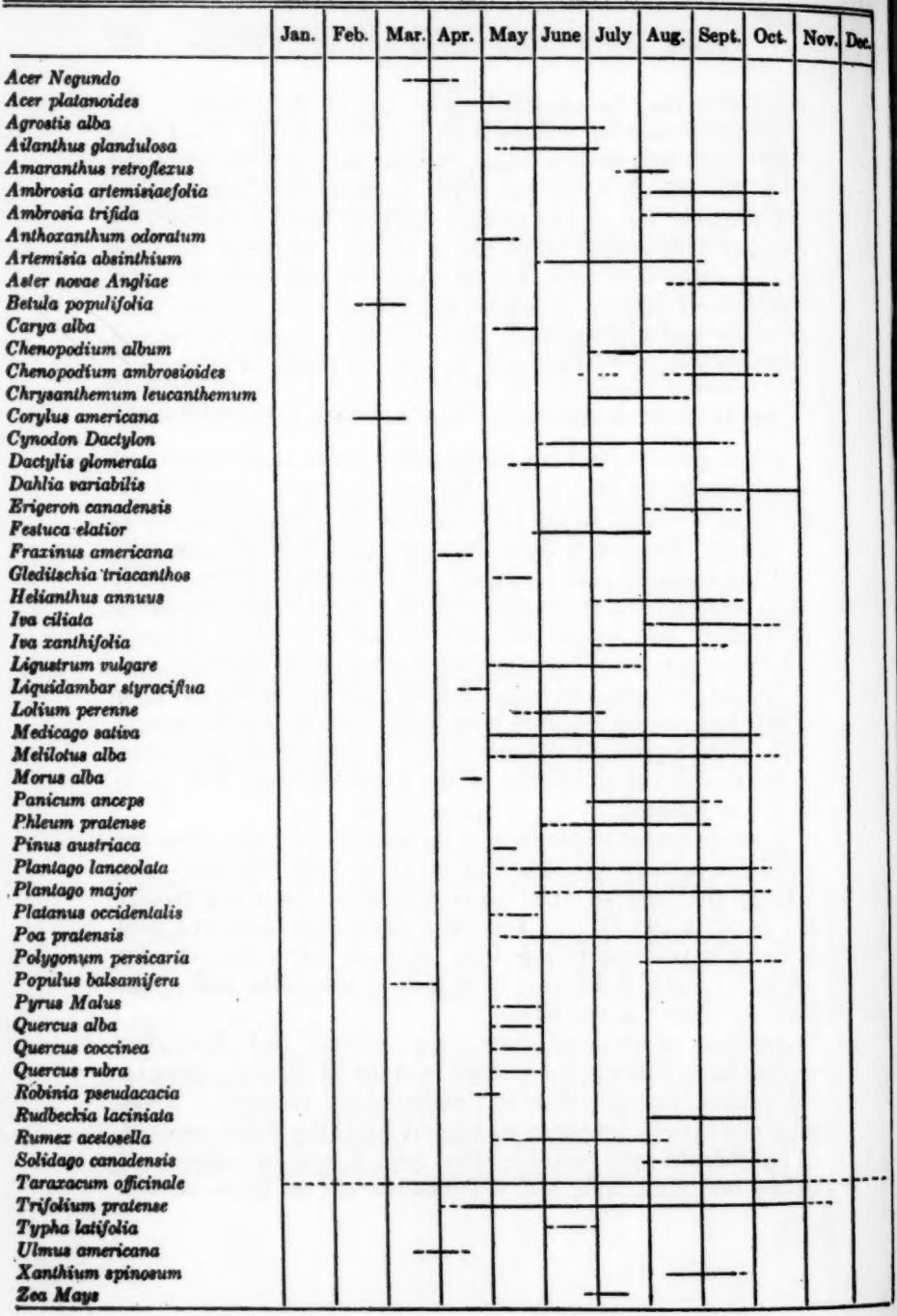
THE EFFECTS OF DIFFERENT REAGENTS ON POLLEN GRAINS

Pollen grains have been tested with a series of chemicals and stains commonly used in botanical microtechnics. Some of the reactions are listed in the synoptical tables. During the course of this investigation it has been ascertained that only the tests with three mineral acids (sulphuric, nitric and hydrochloric acids), three alkalies (ammonia water, sodium hydroxide, and potassium hydroxide), and iodine solutions (iodine water and "Lugol's solution") gave reactions which are of any value for identification. Of staining solutions, safranine, fuchsine, methylene blue, gentian violet, and neutral red have been used. The tests with mineral acids gave in general color reactions. In some cases, mentioned in the tables, the appearance of the whole structure or of parts of it was changed.

In concentrated sulphuric acid the contents and the intine are dissolved; usually the dissolved contents swell and creep out through the pores, or when the swelling takes place very rapidly the pollen grains burst. The color reactions in sulphuric acid are mostly confined to the wall. In cases where the contents showed a reaction different from that of the pollen wall it has been mentioned in the tables.

Artemisia absinthium, *Aster novae Angliae*, and *Helianthus annuus* have, either in the contents or in the oil drops occurring on the surface, certain yellow or orange-colored pigments. These pigments gave in concentrated sulphuric acid the typical reaction of lipochromes; the natural yellow color turned into green and finally into dark blue, and the contents and oil drops showed

RELATIVE TIME AT WHICH VARIOUS POLLENS MAY BE EXPECTED TO BE FOUND IN AIR, BASED ON TIME OF POLLINATING



small blue granules or crystals. This reaction is very distinct and specific for the pollen of these three plants, and it can be used as a mark of identification.

In nitric and in hydrochloric acid the reaction was chiefly one of color, although, as might be expected, swelling took place in a majority of cases. In nitric acid in two cases, *Chenopodium album* and *Liquidambar styraciflua*, an effect upon the pores was visible, the latter standing out in bold relief. Only in a few instances was it possible to conclude from the results obtained the presence of specific chemical compounds.

With alkalis very little difference was observed between the reaction of the pollen grains of different plants. In the tables a color reaction is indicated, not only of the contents but the separate parts of the pollen grains (perine, extine, intine, and pores) sometimes showing different colors.

"Lugol's solution" and iodine water are used in botanical microtechnics as reagents for proteins and as a test solution for starch. Where starch occurs it turns more or less rapidly to dark blue or black. Among the fifty-five kinds of pollen investigated, in thirty-four cases the presence of starch grains could be determined unquestionably. In twenty-one cases the result was negative (see tables). The size of the starch grains varies from very small to large, a distinguishing mark of some value. In some pollens only a very few starch grains can be traced; other pollen grains appear packed with starch. Pollen from dried material in the herbarium failed to show any starch reaction.

The other reagents with which the pollen grains have been tested were the following: ethylic alcohol (95 per cent), acetone, acetic acid, chromic acid, Biuret's and Millon's reagent, vanilline, aniline sulphate, and diphenylamine. In acetic and chromic acid no changes took place, except in one case. It is of interest that in chromic acid (1 per cent) in one case (*Agrostis alba*), a swelling of perine and extine occurred. In the other reagents, except Millon's reagent, no change took place which is worth considering.

In Millon's reagent the pollen grains of *Ambrosia artemisiaefolia*, *Chenopodium album*, *Cynodon Dactylon*, *Dahlia variabilis*, *Quercus*

alba, and *Q. coccinea* gave the positive reaction for proteins. This result is significant for the reason that the effect of proteins is considered to be important in the etiology of hay fever. Six plants out of fifty-five is only about 11 per cent; and of these six plants only two are commonly considered to be hay-fever plants of any great importance, *Ambrosia artemisiaefolia* and *Chenopodium album*.

"ACID NIGROSINE" STAIN

While some reagents (acids or alkalis) may bring out the pores for a short time, the reaction is not as definite as it should be, particularly where it is desirable to determine the presence or absence of lids. It has been found necessary for quick identification to make the pores distinct with the aid of a stain. In general the pores do not appear, or they are not easily distinguished in water preparations. Repeated experiments have demonstrated that nigrosine combined with an acetic solution produces the best results, since it immediately makes distinct the acid characters desired and the reaction persists for the necessary length of time. "Acid Nigrosine" is made as follows: To 100 cc. of a 2 per cent acetic acid solution is added 5 cc. of a saturated and filtered alcoholic (ethyl) solution of nigrosine (Gruebler). After mixing thoroughly by shaking, the reagent is ready for use. This reagent has proved to be a very helpful one. By means of this stain there can be distinguished four different classes of openings in the pollen wall.

(1) Pollen grains with large pores which are covered with a lid.
(2) Pollen grains with large pores without a lid (with a very delicate membrane).
(3) Pollen grains with large and very small pores (the large with or without lids).
(4) Pollen grains with a great number of very small pores.

In case 1, the lids of the pores act as a filter. The acid passes through the lids into the contents while the lids hold back the nigrosine and retain it; the lids therefore appear dark violet.

In case 2, where there is no lid (filter), the very delicate membrane allows the stain to pass through unfiltered and to penetrate the contents of the pollen grain. Instead of the contents stain-

ing evenly, the stain takes a definite outline from the opening to the center of the pollen grain in the form of a cylinder or cone. When the stain has reached the center of the pollen grain as many cylinder- or cone-shaped stained areas can be distinguished as there were openings in the pollen wall. Later the stain may spread throughout the contents of the grain, but not until the cylinders or cones above referred to can be readily distinguished, and it is always possible to identify the number of openings in the pollen wall by counting the number of cylinder- or cone-shaped regions.

In case 3, the lids of the large pores show the same reaction as in case 1, the stain being retained in the lids. At the same time, however, the stain enters through the small pores and slowly penetrates the contents. The contents beneath the large pores remain unstained, and we are able to count in this way the number of the large pores. If the large pores are not covered with a lid the stain will enter through both the large and small pores, but more rapidly through the large ones because of the larger surface. Since the stain penetrates the contents through the large pores more quickly than through the small ones this makes it possible to determine easily the number of the large pores. Later the contents are stained uniformly.

In case 4, with numerous fine pores, the stain will enter the contents at many places and make its way rapidly and uniformly throughout the interior so that in a short time the whole contents are evenly stained. In some cases a light coloring of the pollen wall may take place but only after the staining of the contents. It is necessary, to obtain the best results, that only small quantities of the stain be applied. Only a small drop should be added to the water preparation and the reaction watched carefully. By using this method and watching the reaction it is easy to determine the kind and number of openings in the wall of the different pollen grains investigated thus far.

The only exception to be noted is that of the pollen of *Pinus austriaca*. The use of "Acid Nigrosine" reagent did not at first seem to demonstrate either pores or lids. After a time, however, the air-sacs began to take the stain, finally assuming a dark violet color. Comparing this result with those obtained with

other pollens, it would seem that the tissue of the air-sacs takes the nigrosine in the same way that the lids of the pores in other pollens do. The color is stored in the air-sac tissue while the acid penetrates the contents. It is possible that the tissue of the air-sac should be regarded as nothing but an enlarged lid of a pore which is concealed below the air-sac in the pollen wall. Since pine pollen has two air-sacs we may conclude that most probably the pollen grains of *Pinus austriaca* have two pores covered with lids which have expanded to produce the air-sacs.

Through the coöperation of Dr. H. L. Alexander, of the Washington University School of Medicine, and with the assistance of Mr. L. B. Harrison, a start was made during the summer of 1926 towards a survey of the air in and around St. Louis. By this means, if the work can be continued through several seasons, it is hoped that an accurate knowledge of the prevailing pollens in the air at different times may be obtained.

Various stations were established by Mr. Harrison, and ordinary microscope slides, covered with a film of cotton-seed oil, were exposed for twenty-four hours. These were then brought to the laboratory and examined under the microscope. An attempt was made to use the Cohen dust pump for collecting pollen from the air but the apparatus proved to be too perfect in that it gathered in so much dust with the pollen that the pollen could not be identified. On the whole, no more satisfactory method of getting samples of the pollen in the air than by the exposure of plates covered with some oil or similar adhesive material has been developed.

The observations extended over a period of 115 days (from June 23, 1926, to October 15, 1926). Owing to relative absence of pollen on some days, rain or other disturbing factors, only on 51 days were there positive results. These, with the station from which obtained and the name of the plant, are given below.

Date	Station*	Plant
June 23	1	*Chenopodium ambrosioides*
June 26	2	*Phleum pratense*
June 27	1	*Agrostis alba, Dactylis glomerata*
	3	*Dactylis glomerata, Plantago lanceolata*
June 28	2	*Dactylis glomerata, Agrostis alba*
	3	*Dactylis glomerata, Phleum pratense*
June 29	2	*Phleum pratense, Agrostis alba*
June 30	1	*Poa pratensis, Phleum pratense*
July 1	5	*Poa pratensis*
July 2	5	*Poa pratensis, Agrostis alba*
	1	*Poa pratensis, Festuca elatior*
	2	*Poa pratensis*
July 3	2	*Phleum pratense, Poa pratensis*
	3	*Plantago lanceolata, Phleum pratense*
July 4	2	*Poa pratensis, Agrostis alba, Phleum pratense*
	3	*Phleum pratense*
July 5	1	*Festuca elatior*
July 6	1	*Lolium perenne, Rumex acetosella*
	2	*Poa pratensis*
July 7	1	*Phleum pratense*
	3	*Phleum pratense*
	6	*Rumex acetosella, Agrostis alba*
July 8	2	*Plantago lanceolata, Rumex acetosella*
	3	*Ambrosia artemisiaefolia, Panicum anceps, Festuca elatior*
	6	*Phleum pratense, Poa pratensis*
July 11	1	*Phleum pratense, Festuca elatior*
July 12	2	*Chenopodium ambrosioides*
July 14	6	*Festuca elatior, Chenopodium ambrosioides*
July 15	3, 4	*Chenopodium ambrosioides, Festuca elatior*
July 16	2, 4	*Festuca elatior*
	3	*Chenopodium ambrosioides*
July 19	3	*Festuca elatior*
	4	*Plantago lanceolata, Phleum pratense*
July 20	1, 2	*Chenopodium ambrosioides*
	3	*Festuca elatior*
	6	*Chenopodium ambrosioides, Festuca elatior*
July 23	1	*Zea Mays*
	2	*Festuca elatior*

Date	Station*	Plant
July 24	4	*Phleum pratense*
	6	*Phleum pratense*
July 29	1	*Chenopodium ambrosioides*
	4	*Ambrosia artemisiaefolia*
July 31	1, 2	*Ambrosia artemisiaefolia*
Aug. 1	1	*Ambrosia artemisiaefolia*
Aug. 2	2	*Ambrosia artemisiaefolia*
Aug. 3	6	*Ambrosia artemisiaefolia*
Aug. 4	2	*Ambrosia artemisiaefolia*
	4, 6	*Ambrosia trifida*.
Aug. 5	6	*Ambrosia artemisiaefolia*
Aug. 6	1	*Zea Mays* (?)
Aug. 10	4	*Phleum pratense*
	6	*Chenopodium ambrosioides, Iva ciliata, Iva xanthifolia*
Aug. 11	1	*Chenopodium ambrosioides, Amaranthus retroflexus*
	4	*Ambrosia artemisiaefolia*
Aug. 13	3	*Amaranthus retroflexus*
Aug. 14	6	*Ambrosia artemisiaefolia*
Aug. 15	1	*Ambrosia artemisiaefolia, Amaranthus retroflexus*
	2, 3, 4	*Ambrosia artemisiaefolia*
Aug. 22	2	*Ambrosia artemisiaefolia*
Aug. 24	6	*Chenopodium ambrosioides, Taraxacum officinale*
Aug. 25	1–4, 6	*Ambrosia artemisiaefolia* (great quantities)
Aug. 26	1	*Erigeron canadensis*
	2, 6	*Ambrosia artemisiaefolia*
	3	*Ambrosia artemisiaefolia, Ambrosia trifida*
	4	*Ambrosia artemisiaefolia, Solidago canadensis*
Aug. 28	2, 3	*Ambrosia artemisiaefolia*
Aug. 30	6	*Ambrosia artemisiaefolia*
Sept. 1	1, 3, 4, 6	*Ambrosia artemisiaefolia*
Sept. 7	2, 4	*Ambrosia artemisiaefolia*
	3	*Ambrosia artemisiaefolia, Chenopodium album*
Sept. 17	1, 2, 3, 4	*Ambrosia artemisiaefolia*
Sept. 18	2	*Ambrosia artemisiaefolia, Chenopodium album*
	3, 4, 6	*Ambrosia artemisiaefolia*

Date	Station*	Plant
Sept. 20	3	*Chenopodium album*
	2	*Chenopodium album, Ambrosia artemisiaefolia*
	6	*Ambrosia artemisiaefolia, Solidago canadensis*
Sept. 21	4	*Ambrosia artemisiaefolia, Chenopodium album, Medicago sativa*
Sept. 30	6	*Ambrosia artemisiaefolia* (great quantities)
Oct. 6	3, 4	*Ambrosia artemisiaefolia*
Oct. 7–15		No pollen grains

* Index of Stations: 1, # 4700 McPherson; 2 and 3, Skinker Road in front of Fine Arts Building of Washington University; 4, Skinker Road at University Street Car Line; 5, south of Forest Park, east of Forest Park Highlands; 6, Medical School of Washington University.

According to the frequency of pollen grains occurring on the slides, the more important plants can be arranged as follows:

Pollen of	Number of days occurred	Per cent
Ambrosia artemisiaefolia	24	49
Phleum pratense	12	24
Chenopodium ambrosioides	10	20
Festuca elatior	9	18
Poa pratensis	7	14
Agrostis alba	6	12
Chenopodium album, Plantago lanceolata	4	8
Amaranthus retroflexus	3	6
Ambrosia trifida, Dactylis glomerata, Rumex acetosella, Solidago canadensis, Zea Mays	2	4
Erigeron canadensis, Iva ciliata, Iva xanthifolia, Lolium perenne, Medicago sativa, Panicum anceps, Taraxacum officinale	1	2

DESCRIPTION OF POLLENS

Acer Negundo (*Box Elder*). *No. 50.*[1]—Pollinating from middle of March until middle of April. Tetrahedral, 3 pores without lids; 25–40 μ. Contents granular. The dry pollen taken directly from the anthers is folded and shaped like a grain of rye; in water preparation the pollen grains stretch and become tetrahedral. Color grayish yellow. In "Acid Nigrosine" the contents below the pores stain dark violet. Starch present.

Acer platanoides (*Norway Maple*). *No. 43.*—Pollinating from middle of April until middle of May. Tetrahedral, 3 pores with

[1] Numbers refer to those used in key.

lids; 30 × 38 μ. Contents coarse-grained. The pollen wall very thick and shows distinct layers. Color yellowish gray. "Acid Nigrosine" stains the lids of the pores dark violet. No starch present.

Agrostis alba (*White Bent Grass*). *No. 28.*—Pollinating May and June. Elliptical, 1 pore with a lid; 17 × 24–25 × 32 μ. Contents finely granulated. Pollen wall thick. Extine with very delicate light red-brown shimmer. In water grains appear bean-shaped. Colorless. "Acid Nigrosine" stains first the lids of the pores and later the contents. In chromic acid the wall swells and appears very thick (3.5 μ). Starch present.

Ailanthus glandulosa (*Tree of Heaven*). *No. 45.*—Pollinating middle of May until end of June. Tetrahedral, in water more or less spherical, 3 large pores with lids and numerous fine pores; 20–50 μ. Contents finely granular. Color yellow. "Acid Nigrosine" stains the lids of the pores dark violet. Starch may or may not be present. In ammonia water the fine pores become very distinct.

Amaranthus retroflexus (*Pigweed*). *No. 11.*—Pollinating end of July until middle of August. Spherical, numerous fine pores with lids in the thick wall; 23–25 μ. Color grayish yellow. "Acid Nigrosine" stains the pores dark blue and later the contents become violet. Starch present.

Ambrosia artemisiaefolia (*Common or Lesser Ragweed*). *No. 18.*—Pollinating beginning of August until middle of October. Spherical, 3 pores with lids. Pores placed equatorially. Surface studded with obtuse spines; 17–22 μ. Dry grains appear compressed and therefore elliptical, but in water they stretch and become spherical. Color yellow. "Acid Nigrosine" stains only the lids of the pores dark violet. No starch present. Contents give very weak protein reaction in both Millon's and Biuret's reagent.

Ambrosia trifida (*Great Ragweed*). *No. 27.*—Pollinating August and September. Spherical, 3 pores without lids. Pores placed equatorially. Surface studded with short obtuse spines; 35–42 μ. Color golden. "Acid Nigrosine" stains the pores dark violet. Starch present.

Anthoxanthum odoratum (*Sweet Vernal Grass*). *No. 29.*—Pol-

linating first half of May. Elliptical, almost spherical, 1 pore with lid; 32 × 25–34 × 37 μ. Contents coarse-grained. Color yellowish. "Acid Nigrosine" stains the lid of the pore dark violet. Starch present.

Artemisia absinthium (*Wormwood*). *No. 12.*—Pollinating June until the end of August. Spherical, 3 pores with lids. Spiculated surface with oil drops; 20–28 μ. The dry grains appear elliptical. Color yellow. "Acid Nigrosine" stains the lids of the pores dark violet. No starch present. Sulphuric acid produces in the dissolved contents blue granules and crystals (lipochromes?).

Aster novae Angliae (*New England Aster*). *No. 13.*—Pollinating August until October. Spherical, in water somewhat flat, 3 pores with lids; 24–28 μ; young grains 17–22 μ. The surface is studded with short warty spines, 1.7–3.5 μ in length, covered with numerous oil drops. Color yellow. In "Acid Nigrosine" the lids of the pores swell and stain dark violet. In mature grains the extine separates from the pores and they stand out in bold relief. No starch present. In sulphuric acid the dissolved contents and the oil drops appear blue (lipochromes).

Betula populifolia (*American White Birch*). *No. 7.*—Pollinating end of February to the middle of March. Spherical, 3 pores with lids; 27–32 μ. Color yellow. "Acid Nigrosine" stains the lids of the pores dark violet. Abundant starch present.

Carya alba (*Shagbark Hickory*). *No. 34.*—Pollinating last half of May. Elliptical, 3 pores (sometimes 4) with lids; 38 × 49–63 × 70 μ. Pollen wall thick (3.5 μ). Light grayish yellow. "Acid Nigrosine" stains lids of the pores dark violet. In acetic acid the pollen grains become spherical and show fine granulated contents with large oil drops. Starch present.

Chenopodium album (*Lamb's Quarters*). *No. 26.*—Pollinating July until the end of September. Spherical, numerous small pores without lids; 20–32 μ. Surface smooth, contents coarse-granular. Color dirty yellow. "Acid Nigrosine" stains first the pores dark violet, later the contents. Starch present. Millon's reagent gives a positive result.

Chenopodium ambrosioides (*Wormseed*). *No. 55.*—Pollinating end of August until end of October. Polyhedral, numerous fine pores; 24–28 μ. Surface appears uneven, contents coarse-

granular. Color yellowish gray. "Acid Nigrosine" rapidly stains the contents violet. In acetic acid the pores become distinctly visible. Starch present.

Chrysanthemum leucanthemum (*Ox-eye Daisy*). *No. 47.*—Pollinating July until the middle of August. Tetrahedral, 3 pores with lids; 23–28 μ. Surface studded with spines, contents granulated. "Acid Nigrosine" stains lids of pores dark violet. Color yellowish gray. No starch present.

Corylus americana (*American Hazelnut*). *No. 44.*—Pollinating end of February and first half of March. Tetrahedral, 3 pores with lids; 28–35 μ. Surface smooth, contents granulated. Color yellow. "Acid Nigrosine" stains lids of pores dark violet. Methylene blue stains first the pores dark blue, then the contents. Starch present.

Cynodon Dactylon (*Bermuda Grass*). *No. 1.*—Pollinating June until middle of September. Spherical, 1 pore with a lid; 30–38 μ. Contents granulated, surface smooth. "Acid Nigrosine" stains the lid of the pore dark violet. Color dirty yellow. Starch present. Millon's reagent gives a positive result.

Dactylis glomerata (*Orchard Grass*). *No. 30.*—Pollinating end of May and during June. Elliptical, 1 pore with a lid; 24 × 28–32 × 39 μ. Surface smooth, contents coarse-granular. Color grayish. "Acid Nigrosine" stains first the lids of the pores and then the perine. Abundant starch present.

Dahlia variabilis (*Common Dahlia*). *No. 21.*—Pollinating September until October (first frost). Spherical, 12 to 20 large pores with lids; 28–35 μ. Surface covered with oil drops and studded with sharp-pointed spines 3.5 μ in length. Color yellow. "Acid Nigrosine" stains the lids of the pores dark violet. Starch present. Sodium hydroxide changes the color of the contents from bright red to orange, then light brown and finally to yellow (typical tyrosine reaction). The perine swells. Millon's reagent gives a positive result.

Erigeron canadensis (*Horseweed*). *No. 15.*—Pollinating August and September. Spherical, 3 pores with lids; 16–22 μ. Surface spiny. Color pale yellowish gray. "Acid Nigrosine" slowly stains the lids of the pores violet, gradually becoming darker; later the entire contents become dark violet. No starch present.

Festuca elatior (*Meadow Fescue*). *No. 2.*—Pollinating June and July. Spherical, 1 pore with a lid; 23–30 μ. Colorless. "Acid Nigrosine" stains the lid of the pore dark violet, later the contents light violet. Starch present.

Fraxinus americana (*White Ash*). *No. 38.*—Pollinating second and third week in April. Elliptical, 3, sometimes 4, pores without lids, the pores arranged in a circle around the longer axis; 21 × 24–24 × 32 μ. Surface smooth, contents granular. Color brown. "Acid Nigrosine" stains the contents beneath the pores dark violet. Starch present.

Gleditschia triacanthos (*Honey Locust*). *No. 35.*—Pollinating from the middle until the end of May. Elliptical, almost spherical, 3 pores with lids; 28 × 32–42 × 46 μ. Surface finely granular with oil drops in places. Color light greenish yellow. In "Acid Nigrosine" the lids of the pores swell and stain dark violet. No starch present.

Helianthus annuus (*Common Sunflower*). *No. 14.*—Pollinating July until end of September. Spherical, 3 pores with lids; 30–40 μ. Surface covered with oil drops and studded with sharp-pointed spines, 3.5–7 μ in length. Color dirty yellow. In "Acid Nigrosine" lids of pores swell and stain dark violet. In sulphuric acid the oil drops turn blue (lipochromes?). No starch present.

Iva ciliata (*Rough Marsh Elder*). *No. 17.*—Pollinating August until middle of October. Spherical, 3 pores with lids; 24–28 μ. Surface spiny, wall very thick. Color grayish yellow. "Acid Nigrosine" stains dark violet, first, the lids of the pores, later, the contents. Very few starch grains.

Iva xanthifolia (*Burweed Marsh Elder*). *No. 41.*—Pollinating July until September. Elliptical, 3 pores without lids; 14 × 17–17 × 21 μ. Surface studded with spines. Color grayish yellow. With "Acid Nigrosine" the stain enters the pores and penetrates the contents to the center, forming dark violet-colored cones. Starch present.

Ligustrum vulgare (*Privet*). *No. 54.*—Pollinating May until July. Spherical, 3 (sometimes 4) pores without lids; 24–35 μ. Surface reticulated. Color yellow. Contents granulated. "Acid Nigrosine" stains the contents below the pores dark violet. No starch could be recognized.

Liquidambar styraciflua (*Sweet Gum*). *No. 10.*—Pollinating second half of April. Spherical, 12–20 large pores with lids and numerous fine pores; 35–42 μ. Surface smooth, wall very thick (1.7–3.5 μ). Color yellowish. "Acid Nigrosine" stains the lids of the large pores, and since the stain enters through the small pores, the contents in a short time appear dark violet. If only small quantities of the stain are used the larger pores can easily be detected. In chloral hydrate the structure of the grains becomes distinct, especially the pores and the layers of the wall, and the grains assume a polyhedral shape. Starch present.

Lolium perenne (*Darnel or Rye Grass*). *No. 31.*—Pollinating last week of May until end of June. Elliptical, 1 pore with a lid; 24 × 31–32 × 39 μ. Surface smooth, wall very thick (2.5–3 μ). Color yellowish. Contents granular. Lid of the pore stains dark violet in "Acid Nigrosine." No starch present.

Medicago sativa (*Alfalfa*). *No. 36.*—Pollinating May until October. Elliptical, 3 pores with lids; 31 × 35–42 × 45 μ. Surface reticulated. Color grayish yellow. Contents granular. "Acid Nigrosine" quickly stains the lids of the pores dark violet. No starch present.

Melilotus alba (*Sweet clover*). *No. 48.*—Pollinating May until October. Tetrahedral, 3 pores with lids; 20–28 μ. Surface with cone-shaped projections from the pores. Color greenish yellow. Contents granular. "Acid Nigrosine" stains the lids of the pores at first, later the projections. No starch present.

Morus alba (*White Mulberry*). *No. 6.*—Pollinating second half of April. Spherical, 2 pores with lids; 17–21 μ. Surface smooth. Contents granular. Color light gray-brown. "Acid Nigrosine" acts slowly, staining first the lids of the pores and later the pollen wall. Starch present.

Panicum anceps (*Beaked Panicum*). *No. 3.*—Pollinating early July until September. Spherical, 1 pore with lid (dry pollen grains appear elliptical). The pore stands out in bold relief; 30–38 μ; Surface smooth. Contents granular. Color light yellowish gray. "Acid Nigrosine" stains the lid dark violet. Starch present.

Phleum pratense (*Timothy*). *No. 32.*—Pollinating June until end of August. Elliptical, 1 pore with lid; 25 × 31–33 × 35 μ.

Surface smooth. Contents granular. Color light yellow. "Acid Nigrosine" quickly stains the lids of the pores; later the contents are faintly colored. Starch present.

Pinus austriaca (*Austrian Pine*). *No. 40.*—Pollinating first half of May. Elliptical (swelling in water until almost spherical), with 2 air-sacs and probably with 2 pores; 31 × 50–50 × 70 μ; without air-sacs, 31 × 40–38 × 55 μ. No surface markings except air-sacs. Color light yellow with air-sacs black. "Acid Nigrosine" slowly stains the air-sacs violet. In sulphuric acid the air-sacs show a very fine reticular structure. In acetic acid the grains swell and the wall appears very thick (5.2 μ); the pollen grains become bean-shaped. Contents granular. Starch present.

Plantago lanceolata (*English Plantain or Rib Grass*). *No. 9.*—Pollinating middle of May until end of September. Spherical, 12 pores with lids; 20–28 μ. Surface smooth. Contents coarsely granular. Color yellowish gray. "Acid Nigrosine" stains lids of pores dark violet. Starch present.

Plantago major (*Common Plantain*). *No. 8.*—Pollinating June until end of September. Spherical, 6 pores with lids; 20–25 μ. Surface smooth, wall thick. Contents coarsely granular. Color very pale yellow, almost colorless. "Acid Nigrosine" stains at first the lids of the pores and later the contents. Starch present.

Platanus occidentalis (*Sycamore or Buttonwood*). *No. 51.*—Pollinating in May. Tetrahedral, 3 pores without lids; 20–34 μ. Surface smooth. Contents coarsely granular. Color weak dirty yellow. "Acid Nigrosine" stains at first beneath the pores, later all the contents. Starch grains very small and not in great quantity.

Poa pratensis (*Blue Grass*). *No. 4.*—Pollinating from middle of May until end of September. Spherical, 1 pore with a lid; 31–37 μ. Surface smooth. Contents coarsely granular. Color greenish yellow. In "Acid Nigrosine" the lids of the pores first stain dark violet, whereas the pores themselves appear greenish blue. Later the contents take the color and stain dark violet. No starch present.

Polygonum persicaria (*Knotweed or Lady's Thumb*). *No. 23.*—

Pollinating August and September. Spherical, numerous large pores (more than 20) with lids; 28–53 μ. Surface reticulated. Contents granular. Color very light yellow. "Acid Nigrosine" stains the lids of the pores dark violet. In sulphuric, nitric, and hydrochloric acid the folded structure of the reticulations becomes very distinct. Starch present.

Populus balsamifera (*Balsam Poplar*). *No. 25.*—Pollinating middle of March. Spherical, numerous small pores; 20–40 μ. Surface fine-granular. Contents coarse-granular. Color yellow. "Acid Nigrosine" stains the contents dark violet, the wall light violet. The intine and likewise the contents give a positive reaction for myriophylline; in vanilline-hydrochloric acid, pinkish and purple; in diphenylamine from yellow to pink to brown. Raciborski ('93) has determined myriophylline in the young leaves of *Myriophyllum* (hence the origin of the name). Starch present.

Pyrus Malus (*Common Apple*). *No. 52.*—Pollinating in May. Tetrahedral, 3 pores without lids; 34–35 μ. Surface marked with cone-shaped projections from the pores. Contents coarse-granular. Color pale yellowish gray. "Acid Nigrosine" stains the contents dark violet immediately beneath the pores; later the stain moves toward the center in cone-shaped areas. No starch present.

Quercus alba (*White Oak*). *No. 19.*—Pollinating in May. Spherical, 3 pores with lids which are elongated in cone-shaped projections; 28–34 μ. Contents coarse-granular, containing, in addition to a few small starch grains, globoids with protein crystals. Wall thick. Color yellowish. "Acid Nigrosine" stains the lids of the pores and the projections from the lids dark violet. In potassium hydroxide the globoids become distinct. The contents give a positive protein reaction in Millon's reagent. Starch present.

Quercus coccinea (*Scarlet Oak.*) *No. 49.*—Pollinating in May. Tetrahedral, 3 pores with lids and cone-shaped projections from the pores; 24–35 μ. Surface smooth, wall thick. Contents coarse-granular. In "Acid Nigrosine," at first the lids of the pores stain dark violet, then the projections light violet; later the places beneath the pores take the color. The contents give

the positive protein reaction in Millon's reagent. No starch present.

Quercus rubra (*Red Oak*). *No. 42.*—Pollinating in May. Tetrahedral, 3 pores with lids; 24–35 μ. Surface smooth. Contents coarse-granular. Color dirty yellow. "Acid Nigrosine" stains the lids of the pores dark violet, later penetrating beneath the pores. No starch present.

Robinia pseudacacia (*Common Locust*). *No. 46.*—Pollinating at end of April and early in May. Tetrahedral, 3 pores with lids; 28–41 μ. Surface finely granulated. Color light grayish yellow. "Acid Nigrosine" stains the lids of the pores dark violet. Starch present in very small grains.

Rudbeckia laciniata (*Tall Cone-flower*). *No. 16.*—Pollinating in August and September. Spherical, 3 pores with lids; 20–25 μ. Surface studded with spines about 3.5 μ in length. Color yellow. "Acid Nigrosine" stains the lids of the pores dark violet. No starch present.

Rumex acetosella (*Sheep Sorrel*). *No. 39.*—Pollinating from May until August. Elliptical, 4 pores without lids; 21 × 24–24 × 28 μ. Surface smooth with occasional oil drops. Contents coarsely granular. Color yellow. "Acid Nigrosine" very slowly stains the pores pale violet.

Solidago canadensis (*Canada Golden-rod*). *No. 37.*—Pollinating from August until first half of October. Elliptical, 3 pores with lids; 15 × 21–18 × 24 μ. Surface studded with obtuse spines and covered with oil drops. The spines are arranged in rows parallel to the longer axis. "Acid Nigrosine" stains the lids of the pores dark violet. No starch present.

Taraxacum officinale (*Dandelion*). *No. 22.*—Pollinating all the year. Spherical, 12–20 pores with lids; 35–50 μ. Surface reticulated and studded with short blunt spines. Oil drops also abundantly present. Color gold. "Acid Nigrosine" very slowly stains the pores. Sulphuric acid turns the oil drops blue (lipochromes?). No starch present.

Trifolium pratense (*Red Clover*). *No. 20.*—Pollinating from April until November. Spherical, 3 pores with lids and cone-shaped projections from the pores; 28–41 μ. Surface smooth. Color grayish. "Acid Nigrosine" stains the pores and the lids dark violet, the projections light violet. No starch present.

Typha latifolia (*Common Cat-tail*). *No. 5.*—Pollinating in June. Single pollen grains spherical, 1 pore with a lid with margin, pollen wall penetrated by very fine pores; 24–35 μ; groups from 2–5 pollen grains 35 × 42–49 μ, and 38 × 42–45 μ. Single pollen grains occur rarely; usually they are united in irregular aggregations from 2–5 grains. Surface smooth. Color yellow. "Acid Nigrosine" stains only the lids of the pores dark violet. In sulphuric acid the structure of the extine becomes very distinct and the numerous fine pores can be identified very easily. In contradistinction to *Typha latifolia*, *Typha angustifolia* has only single pollen grains which never occur in aggregations. Size 30–42 μ. Each pollen grain has 1 pore with lid, and numerous fine pores, but the surface is granulated. Starch present.

Ulmus americana (*American Elm*). *No. 53.*—Pollinating middle of March until middle of April. Polyhedral, 5 pores with lids; 25–35 μ. Surface smooth. Contents finely granular. Color grayish yellow. "Acid Nigrosine" stains only the lids of the pores dark violet. No starch present.

Xanthium spinosum (*Burweed or Cocklebur*). *No. 24.*— Pollinating from second week of August until second half of September. Spherical, 3 large pores without lids and numerous fine pores; 22–28 μ. Surface smooth. Contents coarse-granular. Color light brown. In "Acid Nigrosine" the pores and places beneath the pores stain dark violet; the contents stain later. No starch present.

Zea Mays (*Indian Corn*). *No. 33.*—Pollinating latter part of June and first half of July. Elliptical, 1 assymetrically placed pore with a lid; 70 × 75–85 × 88 μ. The differences in length of the axis are not very great but spherical pollen grains occur rarely. Surface smooth. Contents granular. Color light yellow. "Acid Nigrosine" stains first the lids of the pores dark violet, later the pollen wall. Starch present.

KEY

The key is based primarily on the shape of the pollen and number of pores. As subdivisions the presence or absence of lids on the pores and surface markings have been used because they are characteristics and stable. The presence of different projec-

tions, such as spines, warts, etc., is very helpful in identification. For some of the spherical pollen grains it was necessary to take into account the presence or absence of starch as well as the comparative size of the grains. Starch, which can be determined easily, is perhaps the only chemical distinction which is of use. Attention should also be paid to the time of pollination which is of great assistance in some cases.

It may be a disputed point whether the tetrahedral and polyhedral pollen should not be regarded as spherical. While these are actually spherical, departure from this shape is caused by the pores, which by their position produce a three- or many-sided appearance. However, since under practically all conditions the angular shape remains constant and there is no difficulty in recognizing it under the microscope, it seems one of the most readily determined characters for identification.

KEY

I. *Spherical pollen grains, large pores with lids*			
1. Without surface markings			
A. *1 pore*			
a. Single pollen grains			
α. With starch grains 0.5–1.7 μ			
Starch grains 0.5–0.6 μ; pollen grains 30–38 μ.	June–Sept.	*Cynodon Dactylon*	1
Starch grains 0.8–1.1 μ; pollen grains 23–30 μ	June, July	*Festuca elatior*	2
Starch grains 1.1–1.7 μ; pollen grains 30–38 μ	July–Sept.	*Panicum anceps*	3
β. Without starch grains			
Coarse-grained; pollen grains 31–37 μ	May–Sept.	*Poa pratensis*	4
b. Pollen grains aggregated in groups from 2–5, 35 × 42–35 × 49 μ; single pollen grains 24–35 μ	June	*Typha latifolia**	5
B. *2 pores*			
17–21 μ	April	*Morus alba*	6
C. *3 pores*			
27–32 μ	Feb., Mar.	*Betula populifolia*	7
D. *6 pores*			
20–25 μ	June–Sept.	*Plantago major*	8
E. *12 pores*			
20–28 μ	May–Oct.	*Plantago lanceolata*	9
F. *12–20 pores*			
35–42 μ	April	*Liquidambar styraciflua*	10
G. *More than 20 pores*			
23–25 μ	July., Aug.	*Amaranthus retroflexus*	11
2. With surface markings			
A. *3 pores*			
a. Surface spiny			
α. Oil drops on surface			
Spines 1.7–3.5 μ; pollen grains 20–28 μ	June–Aug.	*Artemisia absinthium*	12
Spines 1.7–3.5 μ; pollen grains 24–28 μ	Aug.–Oct.	*Aster novae Angliae*	13
Spines 3.5–7 μ; pollen grains 30–40 μ	July–Sept.	*Helianthus annuus*	14
β. No oil drops on surface			
Spines 0.8–0.9 μ; pollen grains 16–22 μ	Aug., Sept.	*Erigeron canadensis*	15
Spines 3.5 μ; pollen grains 20–25 μ	Aug., Sept.	*Rudbeckia laciniata*	16

b. Surface warty			
Warts 1.2 μ; pollen grains 24–28 μ	Aug.–Oct.	*Iva ciliata*	17
Warts 1.7 μ; pollen grains 17–22 μ	Aug.–Oct.	*Ambrosia artemisiaefolia*	18
c. Surface with cone-shaped projections from pores			
Starch present; pollen grains 28–34 μ	May	*Quercus alba*	19
Starch absent; pollen grains 28–41 μ	Apr.–Nov.	*Trifolium pratense*	20
B. *12–20 pores*			
a. Surface with oil drops and sharp spines; pollen grains 28–35 μ	Sept., Oct.	*Dahlia variabilis*	21
b. Surface with oil drops and short blunt spines; pollen grains 35–50 μ	All the year	*Taraxacum officinale*	22
C. *Numerous pores*			
a. Surface with hexagonal reticulations; pollen grains 28–53 μ	Aug., Sept.	*Polygonum persicaria*	23
II. *Spherical pollen grains, large pores without lids*			
1. Without a surface marking; with numerous fine pores			
A. *3 large pores;* contents coarse; pollen grains 22–28 μ	Aug., Sept.	*Xanthium spinosum*	24
B. *No large pores*			
Pollinating in March; pollen grains 20–40 μ	March	*Populus balsamifera*	25
Pollinating July–September; pollen grains 20–32 μ	July-Sept.	*Chenopodium album*	26
2. With surface markings			
A. 3 pores; surface spiculated; pollen grains 35–42 μ	Aug., Sept.	*Ambrosia trifida*	27
III. *Elliptical pollen grains, large pores with lids*			
1. Without surface markings			
A. *1 pore*			
a. Pore placed laterally; very small starch grains; pollen grains 17 × 24–25 × 32 μ	May, June	*Agrostis alba*	28
b. Pore placed at one end of the longer axis; pollen grains 32 × 35–34 × 37 μ; small starch grains	May	*Anthoxanthum odoratum*	29
c. Pore placed at one end of the longer axis; numerous fine pores in the pollen wall; large starch grains; pollen grains 24 × 28–32 × 39 μ	May, June	*Dactylis glomerata*	30
d. Pore placed at one end of the longer axis; no starch grains; pollen grains 24 × 31–32 × 39 μ	May, June	*Lolium perenne*	31
e. Pore placed laterally; large starch grains; pollen grains 25 × 31–33 × 35 μ	June–Aug.	*Phleum pratense*	32
f. Pore placed laterally; large starch grains; pollen grains 70 × 75–85 × 88 μ	June, July	*Zea Mays*	33
B. *3 pores*			
Starch grains; pollen grains 38 × 49–63 × 70 μ	May	*Carya alba*	34
No starch grains; pollen grains 28 × 32–42 × 46 μ	May	*Gleditschia triacanthos*	35
2. With surface markings			
A. *3 pores*			
Small reticulations; pollen grains 31 × 35–42 × 45 μ	May–Oct.	*Medicago sativa*	36
Obtuse spines; pollen grains 15 × 21–18 × 24 μ	Aug.–Oct.	*Solidago canadensis*	37
IV. *Elliptical pollen grains, large pores without lids*			
1. Without surface markings			
A. *3–4 pores;* pollen grains 21 × 24–24 × 31 μ	April	*Fraxinus americana*	38
B. *4 pores;* pollen grains 21 × 24–24 × 28 μ	May–Aug.	*Rumex acetosella*	39
2. With surface markings			
A. *2 pores; 2 air-sacs;* pollen grains 31 × 50–50 × 70 μ (31 × 40–38 × 55 μ)	May	*Pinus austriaca*	40
B. *3 pores; surface spiny;* pollen grains 14 × 17–17 × 21 μ	July–Sept.	*Iva xanthifolia*	41

V. *Tetrahedral pollen grains*			
A. *3 pores with lids*			
1. Surface smooth			
a. Pollinating in May; contents coarse-grained; pollen grains 24–35 μ	May	*Quercus rubra*	42
b. Pollinating in April; contents coarse-grained; pollen grains 30–38 μ	April, May	*Acer platanoides*	43
c. Pollinating in February and March; contents fine-granulated; pollen grains 28–35 μ	Feb., Mar.	*Corylus americana*	44
d. Pollinating in May and June; contents fine-granulated; pollen grains 20–50 μ	May, June	*Ailanthus glandulosa*	45
2. Surface with markings			
a. Surface fine-granulated; pollen grains 28–41 μ	April, May	*Robinia pseudacacia*	46
b. Surface spiny; pollen grains 23–28 μ	July, Aug.	*Chrysanthemum leucanthemum*	47
c. Surface with cone-shaped projections from pores			
α. Contents fine-granular, starch present; pollen grains 20–28 μ	May–Oct.	*Melilotus alba*	48
β. Contents coarse-grained; no starch present; pollen grains 24–35 μ	May	*Quercus coccinea*	49
B. *3 pores without lids*			
a. Surface smooth			
α. Contents granular; pollen grains 25–40 μ	Mar., Apr.	*Acer Negundo*	50
β. Contents coarse; pollen grains 20–34 μ	May	*Platanus occidentalis*	51
b. Surface with cone-shaped projections from pores; contents coarse; pollen grains 34–35 μ	May	*Pyrus Malus*	52
VI. Polyhedral pollen grains			
1. *With lids*			
A. 5 pores; pollen grains 25–35 μ	Mar., Apr.	*Ulmus americana*	53
2. *Without lids*			
A. 3 large pores; pollen grains 24–35 μ	May–July	*Ligustrum vulgare*	54
B. Numerous fine pores; pollen grains 24–28 μ	Aug.–Oct.	*Chenopodium ambrosioides*	55

* The pollen of *Typha latifolia* is easily distinguished from that of *Typha angustifolia*, the latter having grains always spherical and occurring singly, never in aggregations. Measurements: 30–42 μ. The surface is fine-granulated and the pollen wall is penetrated by numerous fine pores. Each pollen grain has one large pore with a lid.

Synoptical Table

As a complement to the key, the following synoptical table has been prepared. The table may be of some assistance to those wishing to have a summary of the various characters of a particular pollen. Only the more important reactions have been noted, it being superfluous to mention reactions which occur universally and are very well known to every investigator.

Plant	*Acer Negundo*	*Acer platanoides*	*Agrostis alba*	*Ailanthus glandulosa*
Pollinating	Mar. 23–Apr. 4	Apr.–May	May–June	End May–end June
Size	25–40 μ	30–38 μ	17 × 24–25 × 32 μ	20–50 μ
Shape and marks*	t O^1; S smooth; C granular	t O^3 L; S smooth; C coarse	e O^1 L; S smooth; C fine-granulated	t O^3 L o^n; S smooth; C granular
Color	Grayish yellow	Yellowish gray	Colorless	Light yellow
Sulphuric acid	Grayish yellow–golden–brown	Lemon–pale lemon	E pink–purple; P golden–light yellowish; O distinct	Orange–brown; fine pores distinct
Nitric acid	Grayish lemon–yellowish; C swell	No change	PE swell a little, show Str red-brown–delicate purple	Lemon–yellow–greenish yellow; C swell and creep out
Hydrochloric acid	Grayish yellow–lemon–dirty yellow	C swell to double size; PW yellowish; Str distinct	C yellow–colorless; PE golden–delicate greenish yellow	Lemon–dirty yellow; C swell and creep out
Iodine water	Light brown; St black	Dark brown; no starch	Brown; St black	Brown; St black; P yellow
"Lugol's solution"	Brown; St black	Dark brown; no starch	Brown; C and L darker than PW; St black	Brown; St black; P yellow
Ammonia water	No reaction	C swell a little and clear up; granules in the C disappear	Yellow–colorless; C and O^1 Str distinct; P purple	Light yellow; C transparent; Str distinct
Sodium hydroxide	Yellowish, transparent; C swell	C lemon–pale lemon; Str of PW very distinct	Yellow–colorless; P and I yellow; E bright red-brown, distinct	Golden; I red-brown, distinct
Potassium hydroxide	Yellowish, transparent; C swell	C swell threefold, creep out; PW lemon	Same as above	Greenish yellow–yellow–golden; I light red-brown
"Acid Nigrosine"	Parts below the O dark violet	L stain dark violet	L dark violet; later C violet, P light violet	L dark violet

* *Abbreviations used in the Synoptical Table:*
Shape.—e, elliptical; p, polyhedral; s, spherical; t, tetrahedral.
Pores.—O, large pores; o, small pores; L, lid of a pore.
The number of the pores is expressed by an index figure, thus—O^3L, three large pores with lids; O^1, one large pore without a lid; O^n, numerous large pores; o^n, numerous small pores.
Other abbreviations.—C, contents; E, extine; I, intine; P, perine; PW, pollen wall; S, surface; St, starch; Str, structure.

Plant	*Amaranthus retroflexus*	*Ambrosia artemisiaefolia*	*Ambrosia trifida*	*Anthoxanthum odoratum*
Pollinating	End July–first half Aug.	Aug.–Oct.	Aug.–Sept.	May
Size	23–25 μ	17–22 μ	35–42 μ	32 × 35–34 × 37 μ
Shape and marks	s o^n L; S smooth; C coarse	s O^3 L; S obtuse spines equatorially arranged	s O^3; S spiculated	e O^1 L; S smooth; C coarse
Color	Grayish yellow	Yellow	Golden	Yellowish
Sulphuric acid	Lemon–orange–light brown–purple; o distinct	Lemon–light yellow–colorless	Yellow–colorless; PE swell, layers distinct; P at last greenish yellow	Lemon–yellow–light brown; grains swell a little
Nitric acid	Lemon; I very delicate purple	Greenish yellow–light yellow; C swell and creep out	Colorless; P light yellowish; C swell and creep out	Colorless; C swell a little; I lavender and purple; P distinct
Hydrochloric acid	P red-brown–purple	Lemon–golden; C swell	Lemon–deep yellow; C swell and creep out	Yellowish–colorless; PW lemon–bright orange; OL distinct
Iodine water	Light brown; St black	Greenish yellow–brown; no starch	Brown; St black	Light brown; St black; PW dark brown
"Lugol's solution"	Brown; St black; P colorless	Light brown; no starch	Light brown; St black	As in iodine water
Ammonia water	Light brown	Yellowish green; C swell and creep out	Lemon; C shrink	PW lavender
Sodium hydroxide	Light brown; I red-brown	Golden–greenish yellow; C swell and creep out	Greenish yellow–light yellow; C swell and creep out	PW bright orange very distinct
Potassium hydroxide	Light brown; I red-brown	Golden–greenish yellow	Lemon; C swell and creep out	As in sodium hydroxide
"Acid Nigrosine"	o dark violet; C later violet	L dark violet	O dark violet	L stains dark violet

Plant	*Artemisia absinthium*	*Aster novae Angliae*	*Betula populifolia*	*Carya alba*
Pollinating	June–Aug.	Aug.–Oct.	Feb.–Mar.	May
Size	20–28 μ	24–28 μ	27–32 μ	38 × 49–63 × 70 μ
Shape and marks	s O^3 L; S spiny	s O^3 L; S spiny; oil drops	s O^3 L	e O^3 L; S smooth; C coarse
Color	Yellow	Yellow	Yellow	Light grayish yellow
Sulphuric acid	Dirty yellow–grayish blue; PW dirty green, finally light green; C blue granules and crystals	Green–greenish yellow–blue; oil drops blue	Lemon–orange–golden–brown	Orange–lemon–light yellow
Nitric acid	Light yellow–colorless	Almost colorless; C swell and creep out, forming drops on the pores	Lemon–greenish yellow; C swell; P and O distinct	Lemon–light yellow; P lemon; E very delicate pinkish; C swell and creep out
Hydrochloric acid	Entirely decolorized	C swell and creep out; oil drops deep golden	Yellowish green; C swell and creep out	Golden–light yellow; P light brown

Plant	*Artemisia absinthium*	*Aster novae Angliae*	*Betula populifolia*	*Carya alba*
Iodine water	Light brown; L yellow; no starch	Light brown; no starch; oil drops greenish	Black (stuffed with starch); P brown; O yellow	Golden–brown; St black
"Lugol's solution"	Brown; L yellow; no starch	Brown; no starch; oil drops greenish	C black (starch); O light brown	Brown; St black; P golden
Ammonia water	Lemon–light yellow–dirty yellow; C swell	Greenish yellow; C swell a little	Greenish yellow	Yellow; P darker, swells to double size
Sodium hydroxide	Lemon–golden–colorless	Pale yellow–light yellow	Golden; Str distinct	Yellow; P golden; O and layers distinct
Potassium hydroxide	As in sodium hydroxide; oil drops golden	Greenish yellow–light yellow; C swell, become distinct	Yellow; Str distinct	As in sodium hydroxide
"Acid Nigrosine"	L stain dark violet	L stain dark violet	L stain dark violet	L stain dark violet

Plant	*Chenopodium album*	*Chenopodium ambrosioides*	*Chrysanthemum leucanthemum*	*Corylus americana*
Pollinating	July–Sept.	Aug.–Oct.	July–Aug.	Feb.–Mar.
Size	20–32 μ	24–28 μ	23–28 μ	28–35 μ
Shape and marks	s o^n; S smooth; C coarse	p o^n; S smooth; C coarse	t O^3 L; S spiny; C granulated	t O^3 L; S smooth; C granulated
Color	Dirty yellow	Light yellowish gray	Yellowish gray	Yellow
Sulphuric acid	Orange–bright red-brown; C pale yellow; PW bright red-brown	Orange–red-brown–ruby-colored–purple	Lemon–greenish yellow–dirty yellow–colorless	Grayish yellow–orange–light brown
Nitric acid	Bright yellow–pale yellow; C swell a little and creep out	Lemon; o distinct	Colorless	No change
Hydrochloric acid	Yellow	C yellow; I red-brown	Golden–grayish yellow–colorless; S golden oil drops	Yellowish–greenish; C swell and creep out
Iodine water	Brown; St black	Light brown; St black	C yellow; PW brown; no starch	Brown; St black; P light brown
"Lugol's solution"	Dark brown; St black	Light brown; St black	C dark yellow; PW brown; no starch	Golden brown; St black; O golden
Ammonia water	Lemon–yellow–light yellow	Grains shrink; I bright red-brown	Yellow–golden–lemon; C swell and creep out	Golden–yellow
Sodium hydroxide	Golden–light brown (bright)	Lemon–light brown; o distinct	Lemon; C swell and creep out	Lemon–yellow
Potassium hydroxide	Golden–lemon–yellow	Lemon; P bright red-brown; grains swell; o distinct	As in sodium hydroxide	As in sodium hydroxide
"Acid Nigrosine"	o stain at first, later the C	C stain violet very rapidly	L stain dark violet	L stain dark violet

Plant	*Cynodon Dactylon*	*Dactylis glomerata*	*Dahlia variabilis*	*Erigeron canadensis*
Pollinating	June–Sept.	May–June	Sept.–Oct.	Aug.–Sept.
Size	30–38 μ	24 × 28–32 × 39 μ	28–35 μ	16–22 μ
Shape and marks	s O^1 L; S smooth; C granulated	e O^1 L o^n; S smooth; C coarse	s O^{12-20} L; S sharp spines; oil drops	s O^3 L; S spiny
Color	Dirty yellow	Grayish	Yellow	Yellowish gray
Sulphuric acid	Orange–light brown–yellow	Yellowish–pinkish–purple	Orange–light brown–colorless	Light pinkish–yellow
Nitric acid	Yellow–light brown; C swell a little	Yellowish–colorless; C swell and creep out	Greenish yellow–light yellow–colorless; C swell a little	Light yellow–colorless
Hydrochloric acid	Light yellow–colorless	Yellowish; E greenish yellow; I bright red-brown; O greenish yellow	Golden–yellow–light brown; S oil drops	Light yellow–colorless
Iodine water	Brown; St black	Yellow–golden; St black	C brown; PW brown; St black	Light brown; no starch
"Lugol's solution"	As in Iodine water	As in Iodine water	As in Iodine water	As in Iodine water
Ammonia water	Light yellow	Colorless; C swell; P violet–purple	Light brown	Greenish yellow–yellow
Sodium hydroxide	Yellow; grains swell; O swells, appears very distinct; PW orange	C and E yellowish, swell; P bright red-brown; O distinct	Bright red–orange–light brown–yellow; P swells	Lemon–greenish yellow–colorless
Potassium hydroxide	As in sodium hydroxide; O does not swell	As in sodium hydroxide	As in sodium hydroxide	Lemon–greenish yellow
"Acid Nigrosine"	L stains dark violet	L stains at first, later the P	L stain dark violet	L stain at first slowly, later the C

Plant	*Festuca elatior*	*Fraxinus americana*	*Gleditschia triacanthos*	*Helianthus annuus*
Pollinating	June–July	Apr.	May	July–Sept.
Size	23–30 μ	21 × 25–24 × 32 μ	28 × 32–42 × 46 μ	30–40 μ
Shape and marks	s O^1 L; S smooth; C coarse	e O^{3-4}; S smooth; C granulated	e O^3 L; S fine-granulated	s O^3 L; S sharp-pointed spines; oil drops
Color	Grayish	Brown	Light greenish yellow	Dirty yellow
Sulphuric acid	Colorless; E pinkish–purple	Greenish yellow; O distinct	Golden–colorless; P yellow; E pinkish; O distinct	Lemon–greenish yellow; oil drops green–blue

Plant	*Festuca elatior*	*Fraxinus americana*	*Gleditschia triacanthos*	*Helianthus annuus*
Nitric acid	Colorless; C and PW swell; E dark blue; I cherry-colored	Yellow; P light brown; C swell and creep out	Greenish yellow; C swell three or four-fold	Light (pale) yellow; C swell a little
Hydrochloric acid	PW swells to double; P bright red-brown; I lemon-colored	Dirty yellow	Greenish yellow; C swell and creep out; P weak greenish yellow	Yellow (golden)–light (pale) yellow; grains swell and burst
Iodine water	C light brown; St black	Brown; St black	Yellow–chocolate colored; no starch; surroundings of pores not stained	Light brown; no starch
"Lugol's solution"	As in Iodine water	As in Iodine water	As in Iodine water	As in Iodine water
Ammonia water	Colorless; PW bright red-brown and lavender; O distinct	Greenish yellow; fine pores distinct	Greenish yellow–dirty yellow; P colorless	Lemon–colorless; grains swell
Sodium hydroxide	Yellowish; PW swells; P bright red-brown	Greenish golden; fine pores distinct	Lemon; P colorless	Bright lemon; L swell
Potassium hydroxide	C swell; P bright red-brown	Golden–light brown; C swell; fine pores distinct	Greenish yellow–light yellow; C swell and creep out	Lemon–colorless
"Acid Nigrosine"	L stain dark violet, later the C	Parts of C below the O stain dark violet	L swell and stain dark violet	L swell and stain dark violet

Plant	*Iva ciliata*	*Iva xanthifolia*	*Ligustrum vulgare*	*Liquidambar styraciflua*
Pollinating	Aug.–Oct.	July–Sept.	May–July	Apr.
Size	24–28 μ	14 × 17–17 × 21 μ	24–35 μ	35–42 μ
Shape and marks	s O^3 L; S spiny	e O^3; S spiny	p O^3; S smooth; C granulated	s o^n L; S smooth
Color	Grayish yellow	Grayish yellow	Yellow	Yellowish
Sulphuric acid	Lemon–light yellow	Lemon–light yellow; Str of PW distinct	Orange–red-brown–brown	Orange–light brown; P golden
Nitric acid	Light brown; C swell and creep out	Lemon; C swell and creep out	Greenish yellow–dirty yellow; C swell to double size and creep out	Yellow; grains swell; P brown glimmer; o distinct
Hydrochloric acid	Light yellow	Light yellow–colorless; P light pinkish	Light greenish yellow; C swell and creep out	Yellowish–light brown; P light brown
Iodine water	Light yellow; St black	Light brown, later dark brown; St black	Light brown; no St traceable	Yellow; St black
"Lugol's solution"	As in Iodine water	As in Iodine water	As in Iodine water	Golden–brown; St black

Plant	*Iva ciliata*	*Iva xanthifolia*	*Ligustrum vulgare*	*Liquidambar styraciflua*
Ammonia water	Lemon–light yellow–colorless; Str distinct	Yellow–colorless; grains swell; Str distinct	Greenish yellow–dirty yellow	Yellow; grains swell a little; o distinct
Sodium hydroxide	Lemon–greenish lemon; grains swell a little	Greenish yellow–light yellow–colorless	Greenish brown–greenish yellow	Light yellowish–colorless; grains swell
Potassium hydroxide	As in sodium hydroxide	Greenish yellow–colorless; Str distinct	Greenish brown–greenish yellow; P colorless; C swell	Yellow–greenish yellow; grains swell a little
"Acid Nigrosine"	L stain first, later C	Three dark violet cone-shaped stoppers from the O to the centre	Places below the O stain dark violet	Whole grains stain dark violet

Plant	*Lolium perenne*	*Medicago sativa*	*Melilotus alba*	*Morus alba*
Pollinating	May–June	May–Oct.	May–Oct.	Apr.
Size	24 × 31–32 × 39 μ	31 × 35–42 × 45 μ	20–28 μ	17–21 μ
Shape and marks	e O^1 L; S smooth; C granulated	e O^2 L; S small reticulations; C granulated	t O^2 L; S cone-shaped projections from pores; C granulated	s O^2 L; S smooth; C coarse
Color	Yellowish	Grayish yellow	Greenish yellow	Light gray
Sulphuric acid	Light yellow–light brown; O distinct	Pinkish yellow–light brown–grayish; PW yellow	Yellow–light lemon–light yellow	Light brown
Nitric acid	Lemon–violet–purple; P swells	Light brown; C swell; grains burst; PW yellow	Light yellow	P colorless; E pinkish-purple
Hydrochloric acid	Grains swell and burst	Colorless; C swell to double size and creep out	Colorless	C swell and creep out; E light pinkish
Iodine water	Yellow; no St	Brown; no St	Brown; PW yellow; no St	Yellow–brown; St black
"Lugol's solution"	Yellow; P bright red-brown	As in Iodine water	As in Iodine water	Brown; P light brown; St black
Ammonia water	No change	Light yellow; O distinct; grains swell	Lemon–light yellow drops in the C	I pinkish-purple
Sodium hydroxide	Yellow; P bright red-brown; granules very distinct	Greenish yellow–dirty yellow	Lemon–colorless; C swell to double size and creep out	Yellow–colorless; C swell; Str distinct
Potassium hydroxide	Yellow; P red-brown, later light brown; granules distinct	Pale yellow; grains swell threefold and burst; PW chocolate-colored	As in sodium hydroxide	As in sodium hydroxide
"Acid Nigrosine"	L stains dark violet	L stains dark violet	L stains dark violet	At first the L of the O stain; later the P

Plant	*Panicum anceps*	*Phleum pratense*	*Pinus austriaca*	*Plantago lanceolata*
Pollinating	July–Sept.	June–Aug.	May	May–Sept.
Size	30–38 μ	25 × 31–33 × 35 μ	31 × 50–50 × 70 μ; without air-sacs, 31 × 40–38 × 55 μ	20 × 28 μ
Shape and marks	s O^1 L; S smooth; C granulated	e O^1 L; S smooth; C granulated	e O^2 L? S two air-sacs; C granulated	s O^{12} L; S smooth; C coarse
Color	Yellowish gray	Yellow	Light yellow	Yellowish gray
Sulphuric acid	Light brown–orange–golden; PW golden	Light yellow	Orange–flesh-red; P yellow; air-sacs later flesh-red	Orange–pinkish–colorless; PE golden
Nitric acid	Colorless; C clear up	Yellowish–colorless; grains swell to double size	Yellow; P lemon	Golden–yellow; P colorless; E red-brown; L swells
Hydrochloric acid	C colorless	No color reaction; C swell and creep out	C light dirty brown; grains swell to double size	Light red-brown–light brown–colorless; E red-brown; C swell
Iodine water	C dark brown; PW red-brown; St black	Yellow–golden; St black	Lemon–greenish yellow; St black	Golden; St black
"Lugol's solution"	As in Iodine water	Brown; St black	Light brown; St black; grains swell a little	Greenish yellow; St black
Ammonia water	No change	No change	Lemon–dirty yellow	Greenish yellow–colorless; P bright red-brown; O distinct
Sodium hydroxide	Yellow–pale yellow	Light yellow; Str distinct	Yellow; the air-sacs show the color reaction later	Grayish yellow; P red-brown; L swell
Potassium hydroxide	Yellow	No color reaction; O distinct	As in sodium hydroxide, but the color greenish yellow	As in sodium hydroxide
"Acid Nigrosine"	L dark violet	At first the L stains dark violet, later the whole grain	No distinct reaction; after a time the air-sacs stain	L stains dark violet

Plant	*Plantago major*	*Platanus occidentalis*	*Poa pratensis*	*Polygonum persicaria*
Pollinating	June–Sept.	May	May–Sept.	Aug.–Sept.
Size	20–25 μ	20–34 μ	31–37 μ	28–53 μ
Shape and marks	s O^6 L; S smooth; C coarse	t O^2; S smooth; C coarse	s O^1 L; S smooth; C coarse	s O^n L; S hexagonal reticulations; C granulated
Color	Light yellowish	Dirty yellow	Greenish yellow	Light yellow
Sulphuric acid	Lemon–colorless	Lemon–light brown; PW yellow	Orange–yellowish–brown	Greenish yellow–lemon–light brown; surface markings distinct
Nitric acid	Yellow (weak)–colorless	Light brown; C swell	Light yellow; E bright red-brown; grains swell	No color reaction, C swell and creep out; reticulations very distinct
Hydrochloric acid	Colorless; grains burst	Lemon; no change	Light brown; P and E bright red-brown	No color reaction; Str distinct

Plant	*Plantago major*	*Platanus occidentalis*	*Poa pratensis*	*Polygonum persicaria*
Iodine water	Dark brown; O colorless; St black	Brown; very fine and small starch grains which stain black	Light brown; no St	Light brown–greenish yellow; St black
"Lugol's solution"	As in Iodine water	As in Iodine water	Brown; no St	As in Iodine water
Ammonia water	No change	Lemon–dirty yellow; C swell a little	Light yellow; P bright red-brown	Greenish yellow
Sodium hydroxide	Light yellow	Pale yellow–colorless	Light yellow; P and E bright red-brown	Greenish yellow
Potassium hydroxide	Colorless	Lemon; C swell and creep out	As in sodium hydroxide	Greenish yellow–dirty yellow
"Acid Nigrosine"	L stains dark violet, later the C	Places below the O stain dark violet; later C stain	L stains dark violet; later C stain	L stain dark violet

Plant	*Populus balsamifera*	*Pyrus Malus*	*Quercus alba*	*Quercus coccinea*
Pollinating	Mar.	May	May	May
Size	28–40 μ	34–35 μ	28–34 μ	24–35 μ
Shape and marks	s o^n; S fine-granulated	t O^3; S cone-shaped projections from pores; C coarse	s O^3 L; S cone-shaped projections from pores	tO^3 L; S cone-shaped projections from pores; C coarse
Color	Yellow	Yellowish gray	Yellowish	Dirty yellow
Sulphuric acid	Yellowish green–pinkish; P bright yellow	Yellow–lemon–dirty yellow; PW lemon	Orange–red-brown; C yellow	Red-brown–light Indian red
Nitric acid	No reaction	Light yellow	Lemon–light yellow–colorless; C swell threefold and creep out	Lemon–light yellow–colorless; C swell threefold and creep out
Hydrochloric acid	Greenish yellow–colorless, transparent	Colorless; C swell to double size and creep out	Yellow–lemon–greenish yellow	Light yellow; PW darker; O colorless; C swell a little
Iodine water	C brown; St black; I red-brown	Brown; St black	C brown; PW yellow; very few small starch grains, which stain black	Brown; no starch
"Lugol's solution"	As in Iodine water	As in Iodine water	As in Iodine water	As in Iodine water
Ammonia water	PW swells and shows the layers	Colorless	Greenish lemon–greenish yellow	Yellow–lemon–dirty yellow
Sodium hydroxide	No change	Lemon–yellowish; C swell a little	Golden; PW light brown; O colorless	Lemon–dark greenish yellow; PW light brown; O colorless
Potassium hydroxide	Light yellow; grains burst	Lemon–yellowish–colorless	Golden–lemon–yellow; C swell a little; globoids distinct	Lemon–light yellow; C swell a little
"Acid Nigrosine"	C dark violet; PW light violet	Places below the O stain dark violet	L stain dark violet	L stain dark violet, later the places below the O; projections light violet

Plant	*Quercus rubra*	*Robinia pseudacacia*	*Rudbeckia laciniata*	*Rumex acetosella*
Pollinating	May	Apr.–May	Aug.–Sept.	May–Aug.
Size	24–35 μ	28–41 μ	20–25 μ	21 × 24–24 × 28 μ
Shape and marks	s O^3 L; S smooth; C coarse	t O^3 L; S fine-granulated	s O^3 L; S spiny	e O^4; S smooth; oil drops; C coarse
Color	Dirty yellow	Grayish yellow	Yellow	Yellow
Sulphuric acid	Red-brown–light brown	Golden–colorless; P yellow; E light pinkish	Lemon–greenish lemon; PW bluish green	Orange–light purple
Nitric acid	Yellow–light yellow; grains swell and burst	C swell four or five-fold and creep out	Colorless; grains swell a little	Colorless; C swell a little and part creeps out
Hydrochloric acid	Light yellow; C swell	No color reaction; C swell and creep out	Light yellow; Str distinct	Colorless; P yellowish
Iodine water	Light brown; no starch	Light brown–dark brown; St black	Brown; no starch	C yellow; P light brown; St black
"Lugol's solution"	As in Iodine water	Light brown–red-brown; very small starch grains stain black	As in Iodine water	C and P light brown; St black
Ammonia water	Lemon–greenish yellow–dirty yellow	No change	Light yellow–colorless	Grains shrink; Str of P and O distinct
Sodium hydroxide	Golden	Yellow; grains swell and burst	Lemon–greenish yellow–dirty yellow	Yellowish–colorless; S warty
Potassium hydroxide	Golden	Yellow; C swell and creep out	As in sodium hydroxide	Grains swell; S warty
"Acid Nigrosine"	O stain dark violet, later the places beneath the O	O stain dark violet, later the places beneath the O	L stain dark violet	O stain light violet very slowly

Plant	*Solidago canadensis*	*Taraxacum officinale*	*Trifolium pratense*	*Typha latifolia*
Pollinating	Aug.–Oct.	All year	Apr.–Nov.	June
Size	15 × 21–18 × 24 μ	35–50 μ	28–41 μ	35 × 42–38 × 45 μ
Shape and marks	e O^3 L; S obtuse spines; oil drops	s O^{12-20} L; S short blunt spines; oil drops	s O^3 L; S cone-shaped projections from pores	s O^1 L; pollen grains always in aggregations; S smooth
Color	Yellowish	Golden	Grayish	Yellow
Sulphuric acid	Greenish yellow–very light brown	PW purple; oil drops blue	Yellowish	Orange–light brown–light yellow; P light yellow; C light pinkish
Nitric acid	Colorless; C swell and creep out	Light yellow–colorless; C swell and creep out	Light yellow; C swell and creep out	Yellow; C swell and creep out
Hydrochloric acid	Lemon–dirty yellow	C swell; grains burst	C swell and creep out; no color reaction	As in nitric acid

Plant	*Solidago canadensis*	*Taraxacum officinale*	*Trifolium pratense*	*Typha latifolia*
Iodine water	Brown; no starch	Brown; no starch traceable; oil drops bluish green	C brown; no starch traceable	Golden; St black
"Lugol's solution"	As in Iodine water	As in Iodine water	As in Iodine water	As in Iodine water
Ammonia water	Colorless	Pale yellow; grains burst	No change	Golden
Sodium hydroxide	Greenish yellow; Str distinct	Lemon–dirty yellow	Yellowish; C swell and creep out	Golden–light yellow; Str of PW distinct
Potassium hydroxide	Light yellow; Str distinct	Pale yellow	C swell and creep out	As in sodium hydroxide
"Acid Nigrosine"	L stain dark violet	After a long time the O take the color	L dark violet; projections light violet	L stains dark violet

Plant	*Ulmus americana*	*Xanthium spinosum*	*Zea Mays*	
Pollinating	Mar.–Apr.	Aug.–Sept.	June–July	
Size	25–35 μ	22–28 μ	70 × 75–85 × 88 μ	
Shape and marks	p O^4 L; S smooth; C fine-granulated	s O^3 L; S smooth; C coarse	e O^1 L; S smooth; C granulated	
Color	Greenish yellow	Light brown	Light yellowish	
Sulphuric acid	Orange–light yellowish–colorless; PE distinct (Str)	Greenish yellow–brown	Yellow–golden–red-brown; surroundings of the O light yellow	
Nitric acid	Lemon–greenish yellow–colorless; Str of PW very distinct	Light yellow	Light yellow; C swell; O distinct	
Hydrochloric acid	Orange–yellow	Greenish yellow; Str of PW distinct	Yellow–lemon–grayish yellow; C swell; O golden	
Iodine water	Weak yellowish; no starch	Light brown; no starch	C light brown; St black; PW bright red-brown	
"Lugol's solution"	Dark brown, no starch; PE light brown; O distinct	Brown; no starch	As in Iodine water	
Ammonia water	Golden–greenish yellow; O and PW distinct	No change	Grayish yellow; grains swell; O distinct	
Sodium hydroxide	Golden; C swell	Lemon; I red-brown; Str of PW distinct	Yellow–lemon	
Potassium hydroxide	Golden	As in sodium hydroxide	Yellow–lemon; C coarse; P red-brown; O lemon	
"Acid Nigrosine"	L stain dark violet	O and parts of C below stain dark violet	O stains dark violet; PW stains later	

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DETERMINATION OF TOTAL NITROGEN,[1] NITRATE-NITROGEN, AND TOTAL NITROGEN NOT INCLUDING NITRATE-NITROGEN: FURTHER OBSERVATIONS ON A MODIFICATION OF THE OFFICIAL SALICYLIC-THIOSULPHATE METHOD[2]

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INTRODUCTION

The data here presented are in continuation of a series of investigations previously reported (Ranker, '25). These previous data were presented in support of a proposed modification of the official salicylic-thiosulphate method for the determination of total nitrogen including nitrate-nitrogen. The recommended procedure is as follows:

"Place the sample in an 800-cc. Kjeldahl flask; adjust to neutrality or make slightly alkaline; if water is present evaporate *just to* dryness on a water bath under vacuum. Add 35–40 cc. of salicylic acid mixture (1.0 gm. of salicylic acid to 30 cc. of concentrated nitrogen-free sulphuric acid); mix thoroughly and allow to stand for at least an hour with occasional shaking (if organic matter is present, stopper tightly with a rubber cork and allow to stand over night). Add 5 gms. of sodium thiosulphate and heat for 5 minutes with a low flame; cool; add 7–10 gms. of anhydrous sodium sulphate and a pinch of copper sulphate. Digest for an hour at the boiling point after the solution clears; just before the solution solidifies dilute to an estimated volume of 400 cc.; cool completely. Add a small piece of paraffin, 100 cc. of a saturated solution of sodium hydroxide, and a piece of

[1] By the unqualified term "total nitrogen" is meant the sum total of all forms of nitrogen present in the particular sample of material being analyzed.

[2] The second part of a series of investigations carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy in the Henry Shaw School of Botany of Washington University.

mossy zinc; connect immediately to the distillation apparatus and distill 150–200 cc. over into standard acid during a period of 1 hour. Titrate the standard acid to neutrality with standard alkali and calculate the amount of nitrogen present."

In the interest of brevity this proposed method will be referred to as the "modified official method." Certain further applications of the modified official method seemed desirable, and at the same time such an extension would afford opportunity to test its accuracy more extensively. The work has progressed along several lines and certain phases of the investigation are being continued.

In the work previously referred to (Ranker, '25, table 1) it was found that only 92.7 ± .90 per cent of the total nitrogen present was recovered by the modified official method, from samples containing "50 mgs. nitrate-nitrogen plus 0.5 gm. sucrose." In contrast 98.1 ± .64 and 99.5 ± .00 per cent of the total nitrogen were recovered by the comparison method used. Identical samples were used in all cases. From these data it was concluded (page 371) that "if sugar is present in abundance a slight loss of nitrate-nitrogen may occur, due to the reducing action of the sugar." It was concluded further that "this loss would be very slight in actual practice since the nitrate-nitrogen content of plants is small." The last conclusion is based on assumption only. In order to ascertain the true condition that would maintain "in actual practice" some analyses were made to determine the accuracy of the modified official method for samples of plant materials high in sugar and nitrate-nitrogen content. These data are given in table 1.

Strowd ('20) concluded that "the determination of nitrates in plants by finding the difference between the Kjeldahl-Gunning-Arnold method and the Kjeldahl method modified to include nitrates is unsatisfactory." Other workers have noted this difficulty also. Strowd explained this discrepancy on the possible basis that "appreciable amounts of nitrate were apparently reduced without zinc and salicylic acid." However, no data were presented in support of this conclusion (Strowd, '20). In connection with this phase of the problem at least 3 conditions must be recognized:

(1) The inaccuracy of the official salicylic-thiosulphate method,

occasioned by the presence of water in the sample (Ranker, '25), has made impossible a critical comparison with other methods for the determination of nitrate-nitrogen by difference.

(2) The accuracy of the Kjeldahl-Gunning-Arnold method for the determination of "total nitrogen not including nitrate-nitrogen" only, is generally accepted. In the presence of nitrate-nitrogen, however, this method may not be accurate. Such a possibility must be recognized since nitrates are quite universally present in plants; such forms as Selaginella and celery, for example, have a high nitrate content.

(3) The Devarda method is frequently used as a comparison method to test the accuracy of other methods for the determination of nitrate-nitrogen only, in plant materials. Allen ('15) and Davisson ('18) call attention to the possibility that some organic-nitrogen substances may be acted upon in the process. Such a condition would impair the value of the Devarda method for such comparisons and for unqualified determinations of "nitrate-nitrogen" in plant materials.

The above phases of the problem of nitrogen determination were investigated by making certain analyses, as follows:

1—To determine the accuracy of the Kjeldahl-Gunning-Arnold method for the determination of "total nitrogen not including nitrate-nitrogen" only, in nitrate-free samples to which a known amount of nitrate-nitrogen is added.

2—To determine whether or not nitrate-nitrogen only can be quantitatively determined by finding the difference between the modified official method (Ranker, '25) and the Kjeldahl-Gunning-Arnold method.

3—To continue with the study of the influence of certain details of manipulation upon the accuracy of the modified official method in particular.

Materials

The various determinations and analyses were made of samples taken from the various soil and plant materials listed below. Most of these materials are on the plant side and most of them were in solution when used. At the time of sampling, the solutions were free from any precipitate or suspended matter; if not,

they were filtered before samples were taken. All comparable samples were measured into the Kjeldahl flasks (800 cc.) with the same pipette, at the same time and temperature, and all other conditions of making comparable samples were as nearly identical as possible. The numbers assigned to the various materials correspond to the numbers used in the tables and in the text, as indicating the stock materials from which the samples were taken. Though 2 sets of samples may bear the same number they are not necessarily comparable unless they appear in the same table; they may have been measured out at different times and temperatures. The materials analyzed were as follows:

1—*Aspergillus niger.* 2—*Fusarium culmorum.*
Whole cultures, including residual solutions, of the above organisms were cultured under sterile conditions, in 100-cc. flasks containing exactly 25 cc. Pfeffer's nutrient solution to which 1 per cent glucose had been added. The cultures were grown until a vigorous heavy mat had formed. The entire contents of 1 culture flask constituted 1 sample, being transferred without loss to a Kjeldahl flask for determination of nitrogen.

3—*Aspergillus niger.* 5—*Phoma Betae.*
4—*Fusarium culmorum.*
Residual solutions only of the above organisms were cultured, under sterile conditions, in 300-cc. flasks containing 100 cc. of nutrient solution (refer to No. 1 and 2). At time of sampling the cultures were boiled for 5 minutes and filtered. Analyses were made of 25 cc. of the clear filtrate per sample.

6—Tobacco leaves (water extract).
7—Geranium leaves (water extract).
8—Pea leaves and terminals (water extract).
9—Celery leaves and stalks (water extract).
10—Algae mixture, mostly Spirogyra (water extract).
11—Tomato fruits, ripe (water extract).
12—Tobacco leaves and tops (alcohol extract).
13—Geranium leaves (alcohol extract).
14—Pea leaves and terminals (alcohol extract).
15—Celery leaves and stalks (alcohol extract)
16—Tomato fruits, ripe (alcohol extract).

The fresh material of the above plants was ground through a food-chopper; any drippings produced were added; 1 volume of water or 1 volume of 70 per cent alcohol was added; the mixture was boiled (refluxed in the case of alcohol) for 20–30 minutes and filtered hot; 25-cc. samples were taken from the filtrate for analyses.

17—Greenhouse soil plus mushroom compost (water extract).

One kgm. dry material plus 1 L. H_2O; stirred rapidly 4 hrs.; allowe to precipitate; decanted; filtered; 25-cc. samples taken from filtrat

18—Sugar-cane.
19—Sugar-beet.
20—Pea leaves and terminals.

Water extracts of these materials were prepared in the same manner as indicated for Nos. 6–16.

21—Greenhouse soil containing mushroom compost (water extract)
Prepared from same materials and in same manner as No. 17.

22—Mushroom compost only (cold water extract).
Prepared same as No. 17.

23—Mushroom compost (autoclave extraction).
Prepared same as No. 17 except the mixture was autoclaved, not stirred.

24—Mushroom compost, KNO_3.
Twenty-five cc. No. 22 and 74 each, per sample.

25—*Aspergillus niger*, KNO_3.
Twenty-five cc. No. 3 and 74 each, per sample.

26—*Fusarium culmorum*, KNO_3.
Twenty-five cc. No. 4 and 74 each, per sample.

27—Crude peat (water extract).
28—Sphagnum moss (dry) (water extract).
29—*Selaginella apus* (water extract).
30—*Selaginella apus* (expressed sap).
31—*Selaginella apus* (alcohol extract).
32—*Selaginella apus* (water extract).

Prepared same as Nos. 6–16.

Numbers 33–50 refer to various samples of solution cultures of barley, wheat, and peas. These materials were used for qualitative analyses only; the nature and preparation of the samples are indicated in the various tables where the data of their analyses are recorded.

52—Alanin, KNO_3 solution.
Twenty-five cc. alanin solution (1.277 gm. per liter) and No. 74 each, per sample.

53—Asparagin, KNO_3 solution.
Twenty-five cc. asparagin soln. (0.873 gm. per liter) and No. 74 each, per sample.

54—Sugar-cane, KNO_3.
Twenty-five cc. No. 18 and 74 each, per sample.

55—Urea solution.
Two and one-tenths gm. per liter; 25 cc. per sample.

57—Sugar-cane (water extract).
Prepared same as No. 18.

58—Sugar-cane residue, pulp.
Residue from hot-water extractions; washed until free from NO_3 and NH_3.

59—Mushroom compost (water extract).
Prepared same as No. 17.

60—Sugar-beet (water extract).
Prepared same as Nos. 6–16.

61—Sugar-beet residue, pulp.
Residue from hot-water extractions; washed until free from NO_3 and NH_3.

62—Sugar-beet (third water extract).
Third wash water from No. 61, free from NO_3 and NH_3.

64—Urea, KNO_3 solution.
Twenty-five cc. No. 55 and 74 each, per sample.

65—Sugar-beet, KNO_3.
Twenty-five cc. No. 60 and 74 each, per sample.

70—Uric acid solution.
Five gms. per liter; 50 cc. per sample.

71—Taka diastase solution.
Seven and one-half gms. per liter; 50 cc. per sample.

72—Pea seed, germinated.
These seed (Gradus variety) were germinated for 5 days, after which they were mashed in a mortar to a fine pulp in 5 times their weight (dry wt.) of water. This material was free from nitrates; there was a trace of ammonia.

73—Heavy clay-loam soil (water extract).
Prepared same as No. 17.

74—KNO_3 solution.
This solution contained 1.443 gm. of KNO_3 per liter; by analysis, 25 cc. contained $4.79 \pm .005$ mgs. N. In all cases those samples which contained added nitrate-nitrogen received 25 cc. of this solution. The nitrogen content of this solution did not vary throughout the period of these investigations as determined by frequent control analyses of 25-cc. samples.

METHODS

The following methods were used for the determination of nitrogen:

(1) The modified official method as reported by Ranker ('25) for the determination of total nitrogen including nitrate-nitrogen.

(2) A modification of the Devarda method (Ranker, '25) for the determination of nitrate-nitrogen, and for the determination of total nitrogen including nitrate-nitrogen as a comparison method for the modified official method.

(3) The Kjeldahl-Gunning-Arnold method (Association of Official Agricultural Chemists, '25, page 8, No. 24) for the determination of total nitrogen not including nitrate-nitrogen.

The acid and alkali used in titration were standardized against benzoic acid obtained from the U. S. Bureau of Standards (Sample No. 39B). Fiftieth normal acid and alkali were used in all titrations and the normality factors were redetermined frequently to avoid possible errors from this source. All titrations were carried to the complete disappearance of any red tinge as the end-point of methyl red. With N/50 alkali methyl red was found to be much more sensitive than cochineal and the end-point which was used is practically identical with that of cochineal.

Every determination reported in this paper was checked, qualitatively, for the loss of nitrogen at every stage in the process of analysis. The methods of conducting these qualitative control determinations may be grouped under 3 headings:

1. Qualitative tests made during the process of evaporation of the sample under vacuum: All the vapors evolved in this process were passed through a weak solution of sodium bicarbonate and collected in a second flask, both of which are illustrated in fig. 1. The solution thus collected was tested, (A) for the presence of nitrites and nitrates by the diphenylamine test, and (B) for the presence of ammonia by the use of Nessler's reagent.

2. Qualitative tests made following the addition of the salicylic acid mixture to the sample: Any vapors or fumes formed during this process were forced through a weak solution of sodium bicarbonate by blowing gently on the air inlet tube of the device illustrated in fig. 2. In order to obtain as concentrated a solution for the tests as possible, not more than 10–20 cc. of the bicarbonate solution was used as an absorbing solution. The solution thus

obtained was tested, (A) for the presence of nitrites and nitrates by the diphenylamine test, and (B) for the presence of ammonia by the use of Nessler's reagent.

3. Qualitative tests made during the process of acid digestion: The fumes evolved during this process were led through and collected (by the use of a filter pump) in a flask containing approximately 50 cc. of distilled water. The solution thus col-

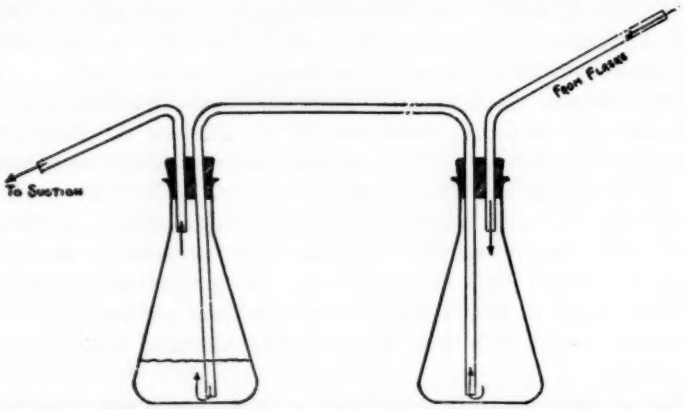


Fig. 1. Arrangement of flasks for the collection of vapors evolved during the evaporation of samples under vacuum and for the collection of fumes evolved during the process of acid digestion.

lected was tested (A) for the presence of nitrites and nitrates by the diphenylamine test and (B) for the presence of ammonia by the use of Nessler's reagent.

The 3 groups of qualitative tests, as indicated above, provide a satisfactory check on the accuracy of each stage of the procedure as recommended in the modified official method. Such qualitative tests are a safeguard against carelessness and should be considered as much a part of quantitative procedure as the actual quantitative determination itself. The data obtained from the qualitative tests are presented as the basis for certain recommendations in regard to details of manipulation and procedure. The above capital letters ("A" and "B") are used throughout the various tables as column headings in the same significance as used above; that is, "A" indicates the results

obtained with the diphenylamine test and "B" indicates results obtained with Nessler's reagent.

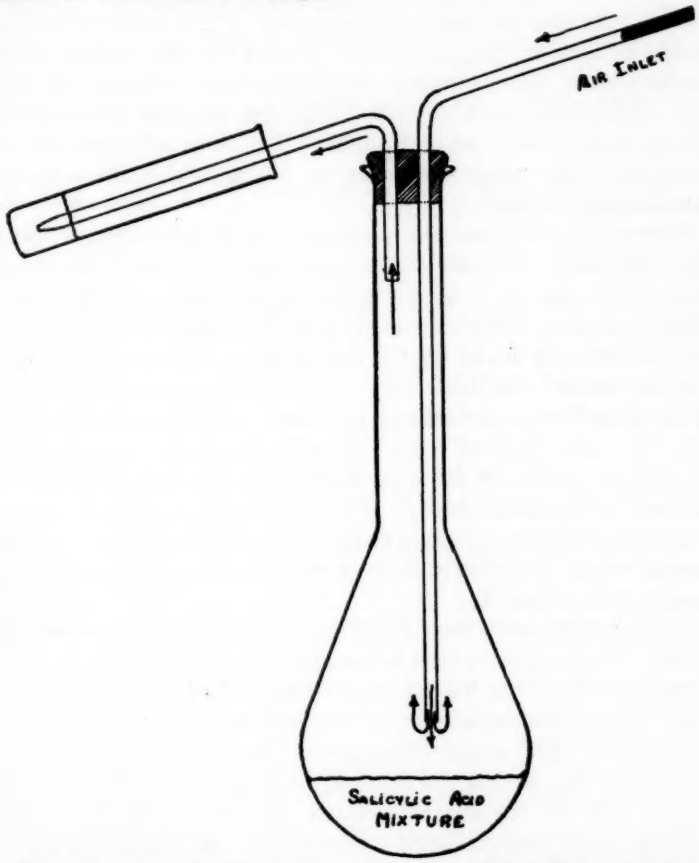


Fig. 2. Method of collecting fumes evolved from samples following the addition of the salicylic acid mixture. The solution collected in the apparatus of figs. 1 and 2 was tested for the presence of nitrites, nitrates, and ammonia.

The evaporation of all samples, which were evaporated, was accomplished on the water bath, under vacuum or as in fig 1.

All probable errors reported in this paper were calculated according to the following formula:

$$\left(E_m = \frac{0.6745\sigma}{\sqrt{n}}\right)$$

EXPERIMENTAL DATA

The prime motive in this investigation has been two-fold: (1) to examine, critically, the accuracy of the modified official method (Ranker, '25), and (2) to extend the application of the method to as many samples of soil and plant materials as time and opportunity would permit. This plan required investigation of the subject from several angles. The data obtained will be presented very much according to the general outline in the introductory remarks.

Before entering into the presentation of data certain terms need definition. The term "vacuum" has reference to an equivalent of not less than 22–23 inches of mercury. By the term "just to dryness" reference is made to that stage in the evaporation process evidenced by the following conditions: the sample has just ceased ebullition and there may or may not be water of condensation in the neck of the flask. By the term evaporation to "ash dryness" the following condition is indicated: the sample, the entire inside of the body of the flask and most of the neck of the flask are dry. Evaporation to "partial dryness" denotes a condition in which some free moisture is present in the sample when the evaporation process is stopped; that is, the sample itself is moist.

In connection with the data of table 1 certain points should be noted. The probable errors are entirely satisfactory; for example, in the case of sample No. 18 (sugar-cane) the nitrogen content as found in 15 determinations by the modified official method is $11.5 \pm .006$. The quantitative results are checked by suitable qualitative tests so that the accuracy of the procedure is definitely known. All of the analyses which were made by these two methods have been included in the table. In practically all of the determinations the accuracy of the modified official method is satisfactory. There are a few cases of rather serious disagreement, however, between the results obtained by the 2 methods. In this connection the determinations for sample No. 6 (tobacco) are noticed at once. This discrepancy seems to exist, also, with the alcohol extract of this material (No. 12), in each case the determination by the Devarda method being low. After several tests this discrepancy was determined to be due to the volatile

TABLE I

ACCURACY OF THE MODIFIED OFFICIAL METHOD FOR TOTAL NITROGEN IN VARIOUS SAMPLES OF BIOLOGICAL MATERIALS

(arithmetical mean and probable error)

Sample determined		No. of trials	Analysis by Devarda method			Analysis by modified official method				
			Nitrogen found (mgs.)	Qual. tests† for loss of N during digestion		Nitrogen found (mgs.)	Qual. tests†, loss of N			
							During evaporation		During digestion	
No.*	Description*			A‡	B‡		A‡	B‡	A‡	B‡
1	Aspergillus niger (whole cult. incl. soln.)	15	2.2 ± .014	—	—	2.3 ± .009	—	—	—	—
2	Fusarium culmorum (whole cult. incl. soln.)	15	2.3 ± .024	—	—	2.4 ± .010	—	—	—	—
3	Aspergillus niger (residual solution only)	17	0.84± .024	—	—	0.83± .005	—	—	—	—
4	Fusarium culmorum (residual solution only)	18	1.4 ± .021	—	—	1.4 ± .013	—	—	—	—
5	Phoma Betae (residual soln.)	15	2.1 ± .011	—	—	2.1 ± .030	—	—	—	—
6	Tobacco (water extract)	15	11.4 ± .027	—	—	12.6 ± .039	—	—	—	—
7	Geranium (water extract)	15	3.7 ± .028	—	—	3.9 ± .020	—	—	—	—
8	Garden pea (water extract)	14	21.6 ± .045	—	—	21.8 ± .021	—	—	—	—
9	Celery (water extract)	15	13.1 ± .030	—	—	13.6 ± .009	—	—	—	—
10	Algae mixture (water extract)	15	0.9 ± .003	—	—	0.9 ± .005	—	—	—	—
11	Tomato fruit (water extract)	15	10.0 ± .018	—	—	10.0 ± .021	—	—	—	—
12	Tobacco (alcohol extract)	15	5.3 ± .028	—	—	5.5 ± .007	—	—	—	—
13	Geranium (alcohol extract)	15	6.0 ± .039	—	—	6.2 ± .011	—	—	—	—
14	Garden pea (alcohol extract)	15	9.0 ± .039	—	—	9.7 ± .015	—	—	—	—
15	Celery (alcohol extract)	15	6.1 ± .027	—	—	6.0 ± .018	—	—	—	—
16	Tomato fruit (alc. extract)	15	15.2 ± .009	—	—	15.2 ± .012	—	—	—	—
17	Greenhouse soil containing mushroom compost (water extract)	15	3.9 ± .013	—	—	3.9 ± .006	—	—	—	—
18	Sugar-cane (water extract)	15	11.4 ± .045	—	—	11.5 ± .006	—	—	—	—
19	Sugar-beet (water extract)	15	10.9 ± .067	—	—	10.7 ± .029	—	—	—	—
20	Garden pea (water extract)	15	29.3 ± .053	—	—	29.3 ± .014	—	—	—	—

* The sample numbers correspond to those numbers in the discussion of materials; the method of preparation and description of the samples, also, are given there.

† The methods of conducting these tests are discussed under "Methods."

‡ The letter "A" is used to designate the diphenylamine test for the presence of nitrites and nitrates; "B," to designate the test with Nessler's reagent for the presence of ammonia.

nature of the nicotine contained in the tobacco material. During the alkaline distillation with Devarda's alloy a large part of the nicotine present is volatilized and driven over into the standard acid sample; some of it is absorbed but much of it passes out into the surrounding atmosphere. Such a loss would contribute toward low results by the Devarda method. This condition is demonstrated by the odor of the nicotine given off and may be determined more or less quantitatively by collecting such vapors in sulphuric acid and analyzing after the Kjeldahl method.

Certain other disagreements exist in the data of table I; for example, in samples No. 6, 9, 12–14. In this connection attention must be called to a rather outstanding fact; in all cases (except sample No. 6 as noted above) in which disagreements are evidenced the relatively low magnitude of the probable error obtained by the modified official method indicates its greater accuracy and reliability. This fact is demonstrated by a consideration of the data for sample No. 9 (13.1 ± .03 as compared with 13.6 ± .009), for sample No. 12 (5.3 ± .028 as compared with 5.5 ± .007), and for sample No. 13 (6.0 ± .039 as compared with 6.2 ± .011). An examination of other sets of determinations reveals the same evidence. The disagreement existing in the case of sample No. 8 does not appear in the case of sample No. 20, although both samples were taken from similar materials. Taken as a whole the data indicate certain points worthy of mention: there is rather satisfactory agreement between the methods; many of the determinations are identical; the data are based on a sufficiently large number of determinations; and the superior accuracy of the modified official method is indicated. Satisfactory results were obtained in the analysis of samples high in sugar content; that is, samples having a high reducing power (samples No. 18 and 19 in table I; samples No. 54 and 60 in table II).

The accuracy of the modified official method is shown, further, by the data of table II. The qualitative results given are considered to be of more value as indicators of the accuracy of the method than purely quantitative data only. The possible errors of distillation and titration are eliminated, thus allowing the distinctive processes of the method (evaporation under

vacuum, addition of the acid mixture, and digestion) to be checked under more exacting conditions. The procedure followed was exactly that previously recommended (Ranker, '25) except that certain of the samples were not adjusted to neutrality (this phase of the subject will be considered in connection with the data of tables v and vi).

TABLE II

ACCURACY OF THE MODIFIED OFFICIAL METHOD FOR TOTAL NITROGEN AS INDICATED BY QUALITATIVE TESTS ON THE PROCEDURE RECOMMENDED*

Sample analyzed		Qualitative tests made† ‖					
		during evaporation		on addition of acid to sample		during digestion	
No.‡	Description‡	A #	B #	A #	B #	A #	B #
21	Greenhouse soil (water extract)	—	—	—	—	—	—
22	Mushroom compost (cold-water extract)	—	—	—	—	—	—
23	Mushroom compost (autoclave extraction)	—	—	—	—	—	—
24	Mushroom compost extract plus KNO_3 solution	—	—	—	—	—	—
25	Aspergillus niger, residual soln. plus KNO_3 soln.	—	—	—	—	—	—
26	Fusarium culmorum, residual soln. plus KNO_3 soln.	—	—	—	—	—	—
27	Crude peat extract (boiled)	—	—	—	—	—	—
28	Sphagnum moss (H_2O extract)	—	—	—	—	—	—
30	Selaginella, expressed sap	—	—	—	—	—	—
31	Selaginella (alcohol extract)	—	—	—	—	—	—
32	Selaginella (water extract)	—	—	—	—	—	—
33	Barley plants (green) plus 10 cc. residual soln. (high in nitrate-N)	—	—	—	—	—	—
34	Same as No. 33 except much lower in nitrate-N	—	—	—	—	—	—
35	Wheat plants (green) plus 10 cc. residual soln. (high in nitrate-N)	—	—	—	—	—	—

TABLE II—Continued

Sample analyzed		Qualitative tests made† ‖					
		during evaporation		on addition of acid to sample		during digestion	
No.‡	Description‡	A #	B #	A #	B #	A #	B #
36	Same as No. 35 except much lower in nitrate-N	—	—	—	—	—	—
37	Pea plants (green) plus 10 cc. residual solution. (high in nitrate-N)	—	—	—	—	—	—
38	Same as No. 37 except much lower in nitrate-N	—	—	—	—	—	—
39	Residual soln. from wheat cultures containing 200 mgs. nitrate-N	—	—	—	—	—	—
40	Same as No. 39 except containing 100 mgs. nitrate-N	—	—	—	—	—	—
41	Same as No. 39 except containing 50 mgs. nitrate-N	—	—	—	—	—	—
42	Same as No. 39 except containing 25 mgs. nitrate-N	—	—	—	—	—	—
43	Same as No. 39 except containing 10 mgs. nitrate-N	—	—	—	—	—	—
44	Same as No. 39 except containing 5 mgs. nitrate-N	—	—	—	—	—	—
45 46 47 48 49 50	These samples were the same as those of Nos. 39–44 except that barley plants were used instead of wheat	—	—	—	—	—	—
52	Alanin plus KNO_3 solution	—	—	—	—	—	—
53	Asparagin plus KNO_3 soln.	—	—	—	—	—	—
54	Sugar-cane extract plus KNO_3 solution	—	—	—	—	—	—
60	Sugar-beet (water extract)	—	—	—	—	—	—

* Ranker ('25).

† The methods of conducting these tests are given under "Methods."

‖ The analysis of each separate sample was repeated 5 times; all 5 tests were in agreement, and as indicated for each sample, respectively.

‡ The sample numbers correspond to those numbers used in the discussion of materials.

"A" is used to designate the diphenylamine test; "B," the test with Nessler's reagent.

A critical examination of certain methods for the determination of nitrate-nitrogen has been undertaken in this investigation. This phase of the subject is still under investigation. It seems desirable, however, to include in this report certain results which were obtained. The accuracy of the Kjeldahl-Gunning-Arnold method (Association of Official Agricultural Chemists, '25, page

TABLE III

ACCURACY OF THE KJELDAHL-GUNNING-ARNOLD METHOD* FOR THE DETERMINATION OF "TOTAL NITROGEN NOT INCLUDING NITRATE NITROGEN"

(Expressed as cc. N/50 HCl neutralized by the ammonia content of the distillate)

Sample determined		Total N present including nitrate-N	Nitrate-N present ‖	"Total nitrogen not including nitrate-N"		
No.†	Description†			Amt. present	Amt. found	% error‡
52	Alanin, KNO_3 soln.	29.3 ± .04	17.1 ± .01	12.2 ± .04		
	sample in solution.				13.5	+ 10.7
	sample in solution.				13.0	+ 6.6
	sample dry, evaporated.				12.7	+ 4.1
53	Asparagin, KNO_3 soln.	27.7 ± .06	17.1 ± .01	10.6 ± .06		
	sample in solution				11.9	+ 12.3
	sample in solution.				12.2	+ 15.1
24	Mushroom compost, KNO_3.	25.4 ± .06	17.1 ± .01	8.3 ± .06		
	sample in solution.				9.0	+ 8.4
	sample in solution.				8.7	+ 4.8
	sample dry, evaporated.				10.8	+ 30.1
54	Sugar cane, KNO_3 soln.	49.5 ± .05	17.1 ± .01	32.4 ± .05		
	sample in solution.				38.5	+ 18.8
	sample in solution.				36.8	+ 13.6
	sample dry, evaporated.				41.9	+ 29.3
25	Aspergillus niger, KNO_3.	17.8 ± .04	17.1 ± .01	0.7 ± .04		
	sample in solution.				0.9	+ 28.6
	sample in solution.				1.1	+ 57.1
	sample dry, evaporated.				2.3	+ 228.6
26	Fusarium culmorum, KNO_3.	18.3 ± .05	17.1 ± .01	1.2 ± .05		
	sample in solution.				1.3	+ 8.3
	sample in solution.				2.1	+ 75.0
	sample dry, evaporated.				3.2	+ 166.7

* Association of Official Agricultural Chemists ('25), page 8, No. 24.

† The sample numbers correspond to those numbers used in the discussion of materials.

‖ This amount of nitrate-nitrogen was added as 25 cc. of solution No. 74; the other component of the sample was nitrate-free as indicated by a negative diphenylamine test.

‡ The basis of these calculations was the amount of nitrogen present (column No. 3) in the form of "total nitrogen not including nitrate-nitrogen."

8, No. 24) for the determination of "total nitrogen not including nitrate-nitrogen" is well established. In the analysis of plant materials (in which nitrate-nitrogen is usually present) the accuracy of this method has been assumed rather generally. Its applicability to samples of plant materials has not always been satisfactorily demonstrated. In attempting to investigate the possibility of estimating nitrate-nitrogen, by finding the difference between the modified official method and the Kjeldahl-Gunning-Arnold method, it seemed imperative to determine the accuracy of the latter method when applied to plant materials. The data of table III cover a part of this work.

The outstanding facts obtained from the data of table III are that all the results are high, and that in the presence of nitrate-nitrogen this method is not accurate when applied to plant materials. Other data, not included in this report, support this conclusion and support the suggestion made by Strowd ('20) that "appreciable amounts of nitrate were apparently reduced without zinc and salicylic acid." In all cases the plant extract component of the respective samples was known to be definitely free from nitrates and nitrites, so that the 25 cc. of solution No. 74 (KNO_3) furnished the only nitrate-nitrogen present. In the case of samples Nos. 25 and 26, the experimental error is high due to the low content of organic nitrogen, and the calculation of the "% error" therefore is subject to the same magnitude of error. As a result these data (Nos. 25 and 26) are not entirely satisfactory. It is of interest to note that in all cases, except for sample No. 52, the inaccuracy of the method is greatly increased when the sample is dry. This result is in sharp contrast to the condition of the official salicylic-thiosulphate method (Ranker, '25) in which case low results were obtained unless the sample was dry.

The first attempts to determine nitrate-nitrogen only, by finding the difference between the modified official method and the Kjeldahl-Gunning-Arnold method, were made on samples similar to those included in table III. Later determinations confirmed the earlier results, but only the latter are included in table IV.

The data of table IV support the same conclusions as do those of table III. This condition would be expected from the situa-

TABLE IV

DETERMINATION OF NITRATE-NITROGEN BY FINDING THE DIFFERENCE BETWEEN THE *MODIFIED OFFICIAL METHOD* AND THE *KJELDAHL-GUNNING-ARNOLD METHOD* *

Sample analyzed		Nitrate-N present in sample (mgs.)‖	Total N found by modified official method (mgs.)	N found by K-G-A. method (mgs.)	Nitrate-N found by difference (mgs.) [compare with column I]	% error**
No.	Description					
65	Sugar-beet, KNO_3	4.8 ± .01	15.5 ± .03	11.3	4.2	—12.5†
				11.6	3.9	—18.8†
				11.8	3.7	—22.8‡
54	Sugar-cane, KNO_3	4.8 ± .01	13.9 ± .01	10.8	3.1	—35.4†
				10.3	3.6	—24.9†
				11.7	2.2	—54.2‡
24	Mushroom compost plus KNO_3	4.8 ± .01	7.1 ± .02	2.5	4.6	— 4.2†
				2.4	4.7	— 2.1†
				3.0	4.1	—14.6‡
25	Aspergillus niger plus KNO_3	4.8 ± .01	5.0 ± .01	0.3	4.7	— 2.1†
				0.3	4.7	— 2.1†
				0.6	4.4	— 8.3‡
26	Fusarium culmorum plus KNO_3	4.8 ± .01	5.1 ± .01	0.4	4.7	— 2.1†
				0.6	4.5	— 6.2†
				0.9	4.2	—12.5‡
52	Alanin, KNO_3	4.8 ± .01	8.2 ± .01	3.8	4.4	— 8.3†
				3.6	4.6	— 4.2†
				3.6	4.6	— 4.2‡
53	Asparagin, KNO_3	4.8 ± .01	7.8 ± .02	3.3	4.5	— 6.2†
				3.4	4.4	— 8.3‡

* Association of Official Agricultural Chemists ('25), page 8, No. 24.

‖ This amount of nitrate-nitrogen was added as 25 cc. of solution No. 74; the other component of the sample was nitrate-free as indicated by a negative diphenylamine test.

** The basis of this calculation was the amount of nitrate-nitrogen present in the sample (column I), that is, 4.8 ± .01 mgm. nitrate-nitrogen.

† Sample in solution, that is, just as made up.

‡ Sample first evaporated to dryness on a water bath under vacuum.

tion as noted in the latter case. There seems to be some correlation between the inaccuracy of the method and the presence of certain reducing substances. Certain results which have been obtained, but which are not included in this report, seem to warrant the belief that with certain modifications these difficulties can be overcome and accurate results obtained in the presence of nitrate-nitrogen. With this accomplished it seems very probable that nitrate-nitrogen only can be quantitatively

determined by finding the difference between the modified official method and a modification of the Kjeldahl-Gunning-Arnold method.

Early in this investigation it became apparent that certain details of manipulation influenced, more or less, the accuracy of the results obtained. In some cases the procedure followed was determined by these factors. The methods of qualitative controls which were developed (discussed under "Methods") made it possible to determine, accurately, the influence of certain details of procedure. Many of the samples were subjected to extreme conditions in order to determine the limits to which certain procedures could be carried. The data obtained from these various studies are given in tables V–IX.

Certain points in regard to the data of table V should be mentioned. The neutrality of the sample is a very important consideration with most of the samples, Nos. 5 and 5b, 29 and 32, 17 and 21. For such samples, this one factor of neutrality would determine the accuracy of subsequent quantitative analyses. The samples just referred to were on the acid side of neutrality prior to neutralization. On the other hand, when the reaction is alkaline a loss of ammonia may occur (sample No. 26a). Another effect of this factor of neutrality should be noted as evidenced by samples Nos. 5b and 5c, 32 and 32a. Sample No. 5b, *Phoma Betae*, having a P_H value of 6.5, could be quantitatively evaporated just to dryness when neutralized, but when evaporated to ash dryness there was a loss of nitrogen (sample No. 5c). On the other hand, sample No. 32a, having a P_H value of 6.8, could be evaporated to ash dryness without a loss of nitrogen. Of all the materials analyzed throughout this entire investigation *Phoma Betae* was the most difficult. When the factors involved (adjustment to neutrality and evaporation just to dryness) were controlled, however, no difficulty was experienced as is shown by the quantitative results obtained (sample No. 5, table I). Sample No. 25 illustrates the opposite extreme; this sample (*Aspergillus niger*) having a P_H value of 3.9 required no adjustment to neutrality even in the presence of added nitrate-nitrogen. These two materials (Nos. 5 and 25) represent the extremes met with. Somewhat similar variations were observed throughout the entire

TABLE V

INFLUENCE OF NEUTRALITY OF THE SAMPLE UPON THE STABILITY OF NITROGEN DURING THE PROCESS OF EVAPORATION

No.*	Sample analyzed and method of treatment	Qual. tests† for loss of N during evaporation‡	
		A #	B #
5	Phoma Betae residual soln., high in NO_3, P_H 6.5 (*not* adjusted to neutrality, evaporated just to dryness)	+	—
5a	Same as No. 5, except evaporation was less vigorous	+	—
5b	Same as No. 5, except sample *adjusted to neutrality*	—	—
5c	Same as No. 5b, except sample evaporated to ash dryness	+	—
29	Selaginella extract, 14 ds. old. P_H *5.5, not neutralized* (evaporated just to dryness)	+	—
32	Selaginella extract, used immediately, P_H *6.8* (evaporated just to dryness)	—	—
32a	Same as No. 32, except sample evaporated to ash dryness	—	—
26	Fusarium culmorum residual soln., plus KNO_3, P_H 8.5	—	—
26a	Same as No. 26, except trace NH_3 added, P_H *8.7*	—	±
17	Greenhouse soil with mushroom compost, H_2O extract, stood in lab. 10 ds., P_H *5.7* (evap. just to dryness)	+	—
17a	Same as No. 17, except extract autoclaved before testing	+	—
21	Sample made from same material as was No. 17, extract analyzed immediately, P_H *7.1*	—	—
25	Aspergillus niger residual soln. plus KNO_3, P_H *3.9* (adjusted to neutrality and evap. just to dryness)	—	—
25a	Same as No. 25, except evaporated to ash dryness	—	—
25b	Same as No. 25, except sample not neutralized (P_H 3.9)	—	—
25c	Same as No. 25a, except sample not neutralized (P_H 3.9)	—	—

* The sample numbers correspond to those numbers used in the discussion of materials.

† The methods of conducting these tests are given under methods.

\# "A" is used to designate the diphenylamine test; "B," to designate the test with Nessler's reagent.

‡ The analysis of each separate sample was repeated 5 times; all 5 tests were identical and as indicated for each sample, respectively.

list of materials used; some required adjustment to neutrality and others did not.

The method of adjusting a sample to neutrality was found to be of extreme importance to accurate procedure. The influence of this factor is apparent from a consideration of the data of table VI.

TABLE VI

INFLUENCE OF METHOD OF NEUTRALIZING THE SAMPLE UPON THE STABILITY OF NITROGEN DURING THE PROCESS OF EVAPORATION

Sample analyzed and method of treatment*	Nitrogen found in sample (mgs.)	Qual. tests† for loss of N during evaporation	
		A #	B #
(a) Shive's nutrient soln., calculated to contain 200 mgs. nitrogen per 950 cc. (*indicator‡ added to sample*, adjusted to neutrality directly)	184.3	+	—
(b) Same as (a), except *indicator was omitted;* adjusted to neutrality by adding a predetermined amount of alkali	202.7	—	—
(c) Shive's nutrient soln., calculated to contain 100 mgs. nitrogen per 950 cc. (*indicator added to sample*, adjusted to neutrality directly)	96.3	+	—
(d) Same as (c), except *indicator was omitted;* adjusted to neutrality by adding a predetermined amount of alkali	101.3	—	—
(e) Shive's nutrient soln., calculated to contain 50 mgs. nitrogen per 950 cc. (*indicator added to sample*, adjusted to neutrality directly)	47.9	+	—
(f) Same as (e), except *indicator was omitted;* adjusted to neutrality by adding a predetermined amount of alkali	48.4	—	—

* All samples were evaporated just to dryness.
† The methods of conducting these tests are given under "Methods."
# "A" is used to designate the diphenylamine test; "B," the test with Nessler's reagent.
‡ The indicator used for these data was brom-cresol purple.

The data of table VI need little comment; the same qualitative results were obtained with other samples and other indicators. *Phoma Betae* samples were extremely difficult to deal with. The choice of indicators that may be used is limited to those which contain no nitrogen, when the indicator is added directly to the sample.

In the procedure for the modified official method (Ranker, '25, p. 371) it is recommended that the sample be neutralized

and then evaporated under vacuum just to dryness. The question of neutrality has been considered in connection with the data of tables v and vi. The study of the factors involved in the process of evaporation was continued, to ascertain the limits of the process and to evaluate the recommendation that the sample be evaporated just to dryness, when applied to various samples. A portion of the results obtained are reported in table vii; the book of data, from which these were taken, contains many more just like those included in table vii. The data included are entirely representative and a sufficient number were taken to show the extremes and the range of variation in the results obtained. In all such analyses the factor of neutrality of the sample was taken care of and controlled; if the sample required neutralization (sample 5, for example) it was adjusted to neutrality by adding a predetermined amount of acid or alkali; if the sample did not require neutralization (samples 33 and 9, for example) it was not neutralized. The data obtained, therefore, may be compared directly in relation to the process of evaporation under vacuum. All of the samples used contained nitrate-nitrogen, either present as shown by a positive diphenylamine test, or as added nitrate-nitrogen, in which case 25 cc. of solution No. 74 was added per sample.

The outstanding fact illustrated by the data of table vii is that the process of evaporation cannot be conducted in any "haphazard" manner. Some samples require rather careful evaporation just to dryness while others may be evaporated to ash dryness and heated for an hour afterward without loss of nitrogen. The most stable material used was No. 3 (*Aspergillus niger*), which with added nitrate-nitrogen (sample No. 25a) could be evaporated to ash dryness with safety. In all cases when the sample was evaporated to partial dryness only, there was a subsequent loss of nitrogen at the time the salicylic acid mixture was added and also during the process of digestion (illustrated by samples No. 33, 35, 37, etc.). The influence of the presence of water in the sample is demonstrated, further, by a consideration of the data for samples Nos. 17 and 17a; these samples were evaporated just to dryness and the acid mixture was added; 5 minutes later a small amount of water was added

TABLE VII

INFLUENCE OF THE EXTENT TO WHICH EVAPORATION IS CARRIED UPON THE STABILITY OF NITROGEN DURING THE PROCESSES OF EVAPORATION, ADDITION OF THE ACID MIXTURE, AND SUBSEQUENT DIGESTION

Sample analyzed*		Qual. tests for loss of nitrogen†					
		during evaporation		on addition of acid to sample		during digestion	
No.‡	Description‡	A #	B #	A #	B #	A #	B #
33	Barley plants plus 10 cc. residual soln. (evap. to *partial* dryness)	—	—	+	—	+	—
33a	Same as No. 33, except evaporated *just to* dryness	—	—	—	—	—	—
33b	Same as No. 33, except evaporated to *ash* dryness	+	—	—	—	—	—
35	Wheat plants plus 10 cc. residual soln. (evap. to *partial* dryness)	—	—	+	—	+	—
35a	Same as No. 35, except evaporated *just to* dryness	—	—	—	—	—	—
35b	Same as No. 35, except evaporated to *ash* dryness	+	—	—	—	—	—
37	Pea plants plus 10 cc. residual soln. (evap. to *partial* dryness)	—	—	+	—	+	—
37a	Same as No. 37, except evaporated *just to* dryness	—	—	—	—	—	—
37b	Same as No. 37, except evaporated to *ash* dryness	+	—	—	—	—	—
17	Greenhouse soil plus mushroom compost, H_2O extract (evap. just to dryness; acid mixture added; 5 minutes later H_2O added)	—	—	+	—	+	—
17a	Same as No. 17, except H_2O added 24 hrs. after acid mixture	—	—	+	—	+	—
17b	Same as No. 17, except evap. to *ash* dryness, no water added	+	—	—	—	—	—
20	Garden pea, water extract (evap. *just to* dryness)	—	—	—	—	—	—
20a	Same as No. 20, except evaporated to *ash* dryness	+	—	—	—	—	—
5	Phoma Betae residual soln. (evap. *just to* dryness)	—	—	—	—	—	—

TABLE VII—Continued

Sample analyzed*		Qual. tests for loss of nitrogen†					
		during evaporation		on addition of acid to sample		during digestion	
No.‡	Description‡	A #	B #	A #	B #	A #	B #
5a	Same as No. 5, except evaporated to *ash* dryness	+	—	—	—	—	—
5b	Same as No. 5, except evap. to *partial* dryness	—	—	+	—	+	—
26	Fusarium culmorum residual soln. plus KNO_3, evap. *just to* dryness	—	—	—	—	—	—
26a	Same as No. 26, except evaporated to *ash* dryness	—	—	—	—	—	—
26b	Same as No. 26, except evap. to *partial* dryness	—	—	+	—	+	—
25	Aspergillus niger residual soln., plus KNO_3, evap. *just to* dryness	—	—	—	—	—	—
25a	Same as No. 25, except evap. to *ash* dryness	—	—	—	—	—	—
25b	Same as No. 25, except evap. to *partial* dryness	—	—	+	—	+	—
9	Celery, water extract, evap. *just to* dryness	—	—	—	—	—	—
9a	Same as No. 9, except evap. to *ash* dryness	—	—	—	—	—	—
24	Mushroom compost extract plus KNO_3, evap. *just to* dryness	—	—	—	—	—	—
24a	Same as No. 24, except evap. to *ash* dryness	—	—	—	—	—	—

* The analysis of each separate sample was repeated 5 times; all 5 tests were identical and as indicated for each sample, respectively.
† The methods of conducting these tests are given under "Methods."
‡ The sample numbers correspond to those numbers used in the discussion of materials.
# "A" is used to designate the diphenylamine test; "B," the test with Nessler's reagent.

and there was a loss of nitrogen; 24 hours later a small amount of water was added to duplicate samples and there was a similar loss of nitrogen. Special attention is called to sample No. 9

(celery). This material was very high in nitrate-nitrogen, second only to sample No. 32 (*Selaginella apus*). Both of these samples required no neutralization and could be evaporated to ash dryness without a loss of nitrogen (since the results were identical the data for sample No. 9 only are reported in this connection).

A special phase of the process of evaporation is illustrated by such materials as whole green plants. When the sample involved contains whole green plants, plus about 10 cc. of the nitrate-containing residual solution, considerable difficulty is experienced in evaporation under vacuum: (1) the colloidal complex of the plant does not give up its water content rapidly in the humid atmosphere inside the flask; (2) the small amount of moisture present in the sample does not evolve sufficient steam to break down effectively the plant structure; (3) the steam evolved under such conditions does not heat the flask sufficiently to prevent condensation and consequent run-back. The semi-equilibrium obtained, however, is such that evaporation may be accomplished in this manner but the time required is too long for efficiency. It required 3–6 hours to evaporate the sample to partial dryness under vacuum, 6–8 hours to evaporate it just to dryness under vacuum, and 7–10 hours, to full dryness under vacuum. These time intervals refer only to the particular kind of sample mentioned above, namely, whole green plants plus about 10 cc. of the nitrate-containing residual solution. Furthermore, it is difficult to judge, under these conditions, when the sample is evaporated just to dryness (sample No. 35, table VIII); if carried on just past this stage there is a loss of nitrogen (sample No. 35a), especially if the sample is high in nitrate-nitrogen. These difficulties may be overcome in one of two ways: (1) by the addition of an appreciable amount (50–100 cc.) of distilled water, sufficient to evolve steam enough to break down completely the plant structure and organization; (2) by the use of some such ventilation device as that illustrated by fig. 3, by which a continuous stream of dry air is used to carry off the vapors from the sample. This latter method is recommended. With this device there was no loss of nitrogen from any sample of whole green plants, even when large amounts of nitrate-nitrogen were added. Some of the data obtained from

this study are reported in table VIII, and are representative and typical of all the data obtained.

Certain points in connection with the data of table VIII are worthy of mention. The time periods required to evaporate the samples under vacuum have been given, namely: to evaporate the samples, under vacuum, to partial dryness, 3–6 hours; just

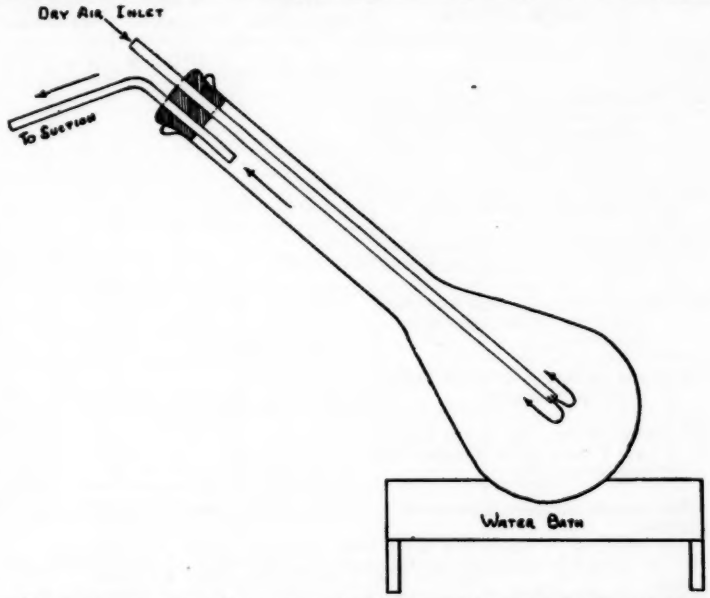


Fig. 3. Arrangement of apparatus for the evaporation of whole green plants by dry-air ventilation.

to dryness, 6–8 hours; to full dryness, 7–10 hours. In contrast, the time periods required to evaporate similar samples to comparable stages of dryness, by using the special ventilation device (fig. 3), were 10–15 minutes, 12–20 minutes, and 15–25 minutes, respectively. When this latter method was used to evaporate such samples there was no loss of nitrogen in any of the tests made. Sample No. 35c, for example, was heated for 1 hour after it was completely dry, with no loss of nitrogen.

Very early in this investigation it was noticed that certain irregular results in titration were obtained. For example, in the

TABLE VIII

INFLUENCE OF THE METHOD OF DRYING WHOLE GREEN PLANTS UPON THE STABILITY OF NITROGEN DURING THE PROCESSES OF EVAPORATION, ADDITION OF THE ACID MIXTURE, AND SUBSEQUENT DIGESTION

Sample analyzed		Qual. tests for loss of nitrogen†					
		during evaporation‡		on addition of acid to sample†		during digestion‡	
No.*	Description*	A #	B #	A #	B #	A #	B #
35	Wheat plants plus 10 cc. of residual solution high in nitrate-nitrogen *evaporated under vacuum just to dryness*	—	—	—	—	—	—
35a	Same as No. 35 except *evaporated under vacuum to full dryness*	+	—	—	—	—	—
35b	Same as No. 35 except *evaporated under vacuum to partial dryness*	—	—	+	—	+	—
35c	Same as No. 35 except *evaporated just to dryness by a special ventilation device***	—	—	—	—	—	—
35d	Same as No. 35c except evaporated to full dryness	—	—	—	—	—	—
35e	Same as No. 35c except heated for 1 hour after sample was completely dry	—	—	—	—	—	—

*The sample numbers correspond to those numbers used in the discussion of materials.

† The methods of conducting these tests are given under "Methods."

"A" is used to designate the diphenylamine test; "B," the test with Nessler's reagent.

‡ The same results were obtained for barley and pea plants, samples No. 33, 34, 37, 38, and for wheat sample No. 36. Each test was repeated 5 times for each separate sample and the same results were obtained each time.

** See fig. 3.

simple determination of nitrogen present in a known solution of ammonium sulphate, 2 each of the 6 titrations consistently required more alkali to titrate the excess acid to neutrality than did each of the other 4; also, these same 2 flasks contained a more or less milky distillate that was quite distinctive from the crystal-clear distillate of the others. After several attempts to locate the cause of this difficulty it was noticed that whenever a certain grade of rubber tubing was used, to connect the condenser bulb with the block tin condenser tube of the distillation apparatus,

TABLE IX

INFLUENCE OF RUBBER TUBING CONNECTIONS* UPON THE QUANTITATIVE DISTILLATION OF AMMONIA INTO STANDARD ACID

Sample analyzed and nature of rubber tubing connections used		Cc. N/50 acid neutralized by ammonia contained in distillate	Error (cc. N/50 acid)
No.†	Description		
11	Tomato fruits, water extract		
	Distillation through connection No. 2; average of 4 tests‖	39.1 ± .08	
	Distillation through connection No. 1‡ used on condenser bulb No. 3	37.6	—1.5
9	Celery, water extract		
	Distillation through connection No. 2; average of 3 tests‖	51.9 ± .04	
	Distillation through connection No. 1‡		
	used on condenser bulb No. 3	50.9	—1.0
	used on condenser bulb No. 4	50.0	—1.9
17	Greenhouse soil and mushroom compost, H_2O extract		
	Distillation through connection No. 2; average of 4 tests‖	17.2 ± .02	
	Distillation through connection No. 3# used on condenser bulb No. 5	12.7	—4.5
73	Heavy clay loam soil, water extract		
	Distillation through connection No. 2, average of 4 tests‖	3.2 ± .03	
	Distillation through connection No. 1‡		
	used on condenser bulb No. 1	2.6	—0.6
	used on condenser bulb No. 2	2.9	—0.3
	used on condenser bulb No. 3	2.9	—0.3
	used on condenser bulb No. 4	2.6	—0.6
18	Sugar-cane, water extract		
	Distillation through connection No. 2; average of 5 tests‖	44.7 ± .06	
	Distillation through connection No. 1‡		
	used on condenser bulb No. 1	43.9	—0.8
	used on condenser bulb No. 2	43.1	—1.6
	used on condenser bulb No. 3	42.9	—1.8
	used on condenser bulb No. 4	40.9	—3.8
	used on condenser bulb No. 5	44.4	—0.3

* As used in this table, the term "connection" refers only to that length of rubber tubing used to connect a glass condenser bulb to a block tin condenser tube of the Kjeldahl distillation apparatus.

† The sample numbers correspond to those numbers used in the "Discussion of materials."

‡ Connections No. 1 were such that about 6 cm. of the total length was exposed to ammonia vapors during distillation. Before use the rubber was extracted once with N/10 NaOH, washed in dilute HCl, and rinsed thoroughly. The rubber tubing used was the ordinary white laboratory grade, thick-walled, cloth-wrapped (A. H. Thomas' No. 8830).

‖ Connections No. 2 were such that not more than 0.5 cm. of the total length was exposed to ammonia vapors during distillation. Before use the rubber was treated as in the case of connections No. 1. The rubber tubing used was A. T. Thomas' No. 8834: black gum and sulphur; steam-cured; thick-walled; smooth bore; of fairly good quality. These connections were adopted for all subsequent work.

Connections No. 3 were the same as connections No. 1 except that about 20 cm. of the total length was exposed to ammonia vapors during distillation.

these milky distillates were obtained. In such distillates the calculated nitrogen content was invariably low. The particular grade of rubber tubing which gave these results was that described in the footnote to table IX and was used in making the "connections No. 1" as indicated. When extracted with N/10 sodium hydroxide a yellow-green sodium sulphide mixture was obtained even after 6 such extractions. This grade of rubber is entirely unsatisfactory for such connections. A better grade of rubber tubing was obtained; this is described in the footnote to table IX and was used to make "connections No. 2" as indicated. (Note that the total length of rubber exposed to the ammonia fumes was not more than .5 cm.). The first extraction of this rubber with N/10 sodium hydroxide produced the characteristic yellow-green sodium sulphide mixture but all subsequent extractions were free from this material. Six check distillations of ammonia, through connections No. 2, produced a probable error of + .0045; these results were entirely satisfactory and this grade of rubber tubing was adopted for all subsequent work. Certain few analyses were made to illustrate the influence of these different rubber connections and these data are given in table IX.

General Discussion and Conclusions

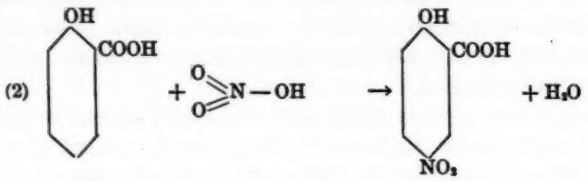
There are certain aspects of the data presented which require some discussion and which require correlation with the data previously reported (Ranker, '25) and with the larger aspects of the determination of nitrogen. In the determination of any form of nitrogen it would seem logical, first, to obtain a method which is accurate for the determination of total nitrogen. Two methods are used generally: (1) some modification of the Devarda method, and (2) the official salicylic-thiosulphate method (Association of Official Agricultural Chemists, '25, p. 9, No. 29). The first method is time-consuming; there is a preliminary distillation with Devarda's alloy, an acid digestion, and a second alkaline distillation. The second method is not accurate when applied to most plant materials in the presence of water (Ranker, '25). This method, however, is relatively rapid when compared with the Devarda method. It is considered that the inaccuracies

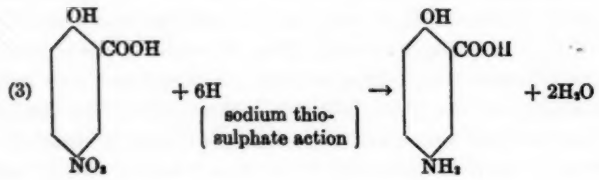
of this second method (official salicylic-thiosulphate method) have been overcome, with no loss of rapidity, by the procedure for the modified official method (Ranker, '25). The data reported in this paper are offered in further support of the accuracy of this method.

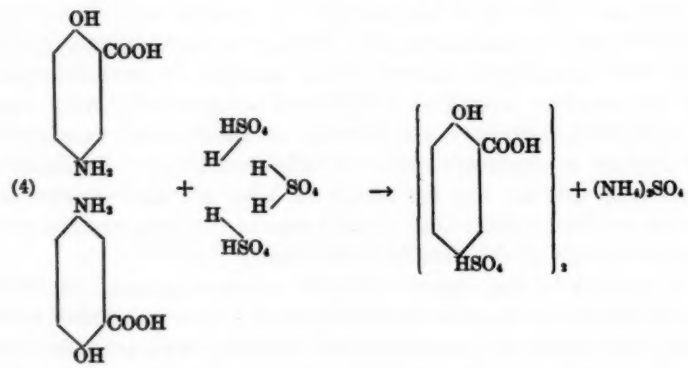
Certain aspects of the chemistry of the modified official method should be considered at this point. Roughly, the nitrogen content of all materials may be divided into 2 classes: (1) organic, and (2) inorganic. Either of these may have an acid or basic reaction. The data of this investigation show that the acid or basic nature of the various forms of nitrogen, as such, does not seem to influence the stability of the total nitrogen contained in the sample. On the other hand, the reaction of the sample seems to determine the stability of its nitrogen-content in many cases. Both acid and basic forms of nitrogen may be present in rather equal proportions in the same sample. Such an occurrence may explain why it is necessary to adjust certain samples to neutrality prior to evaporation. Such an adjustment to neutrality may exert a stabilizing influence, due directly to the relative decrease of hydrogen- or hydroxyl-ions. It may exert, also, an indirect influence, due to the formation of new nitrogen compounds which are more stable than those forms of nitrogen present prior to adjustment of the sample to neutrality.

In regard to the organic nitrogen which is present, it would be transformed into ammonia-nitrogen very much as would occur with the Kjeldahl-Gunning-Arnold method and need be considered no further here. The inorganic nitrogen content of a sample, however, presents a different situation. Of the various forms of inorganic nitrogen, nitrate-nitrogen commands most attention. Given the proper condition of dryness (evaporation just to dryness) for a nitrate-containing sample, the reduction of nitrate-nitrogen to ammonia-nitrogen may be considered to take place somewhat as follows, with the use of salicylic acid:

(1) $NaNO_3 + H_2SO_4 \rightarrow NaHSO_4 + HNO_3$







By equation (2) it is evident that nitric acid is responsible for the nitrification of the salicylic-acid molecule. The nitric acid is obtained from the nitrates present in the sample (equation 1) by the action of the sulphuric acid which is present in the salicylic-acid mixture (1.0 gm. salicylic acid to 30 cc. sulphuric acid). The sulphuric acid plays another important rôle. The quantitative completion of equation (2) and equation (3) toward the right depends upon the loss of water. For each atom of nitrogen thus combined 3 molecules of water are formed. This water is removed from the reaction by the desiccating action of the sulphuric acid. The reactions (equations 2 and 3), thereby

continue to quantitative completion and there is no loss of nitrogen, providing the sample was first evaporated to dryness (Ranker, '25). The sulphuric acid functions in yet another manner, namely, in the digestion of the amino-salicylic-acid molecule and the formation of ammonium sulphate (equation 4).

The various steps in the procedure recommended for the modified official method have been reinvestigated and the results obtained do not warrant any alterations in the procedure. Certain qualifying conditions must be recognized, however: (1) some samples must be adjusted to neutrality prior to evaporation while other samples require no such adjustment; (2) some samples must be carefully evaporated just to dryness and other samples may be roughly evaporated to ash dryness without a loss of nitrogen (the particular chemical or physical complexes responsible for these differences are not known); (3) in the distillation process paraffin may be omitted in some cases but must be present in others to prevent foaming. In just which cases it is safe to take advantage of these qualifying conditions, in order to simplify any step in the procedure, must be decided by the individual investigator using the method. Inasmuch as these factors (neutralization and evaporation just to dryness) are essential to the accurate determination of total nitrogen in some samples they cannot be omitted from the general procedure recommended (Ranker, '25, p. 371).

The above considerations lead directly to a consideration of the importance of the qualitative tests. Without some such tests it is impossible to control, accurately, the various factors involved in the quantitative determination of nitrogen by any of the acid digestion methods, impossible to know, definitely, that there has or has not been a loss of nitrogen in the process, and very difficult to locate any source of error that might exist. It is strongly recommended that suitable qualitative tests (those used in this investigation have been found satisfactory) be considered as an integral part of nitrogen-determination methods whenever it is at all feasible to use them. An example or two might be of value to illustrate this suggestion: (1) certain of the factors contributing to the inaccuracies of methods investigated by Mitscherlich and Herz ('09) could have been definitely located

and corrected in this manner; (2) had such tests been used Strowd ('20), using samples containing plant materials plus nitrate-nitrogen in solution, probably would have detected the inaccuracy of the "Kjeldahl method modified to include nitrate" under such conditions; (3) the use of such qualitative tests would have deterred Gallagher ('23, p. 67) from his theoretical denunciation of the principle of reduction of nitrates in acid medium; (4) the data presented in this report are filled with instances in which all sorts of results would have been obtained had not the quantitative data been checked with qualitative tests on the procedure used. Other examples of a similar nature are abundant. It is fortunate, indeed, that the various methods for the determination of nitrogen are so well adapted to the use of qualitative control tests for the loss of nitrogen. It is unfortunate that these qualitative tests are so seldom used.

One other aspect of this investigation should be mentioned at this time, namely, the determination of nitrate-nitrogen. Consideration of this subject will be restricted to the determination of nitrate-nitrogen by finding the difference between the Kjeldahl-Gunning-Arnold method and the modified official method (a separate report is being prepared on certain phases of the determination of nitrate-nitrogen in plants by the Devarda method). The inaccuracies of the official salicylic-thiosulphate method (Association of Official Agricultural Chemists, '25, p. 9, No. 29) in the presence of moisture have prevented the determination of nitrate-nitrogen by finding the difference between that method and the Kjeldahl-Gunning-Arnold method. With the more accurate procedure of the modified official method such a determination of nitrate-nitrogen was thought possible. The inaccuracies of the Kjeldahl-Gunning-Arnold method when applied to samples containing nitrates (data of table III), however, prevent this possibility. The data presented in this report include only negative results in this direction. Certain tentative modifications which have been tried, however, indicate the possibility of perfecting the Kjeldahl-Gunning-Arnold method to the point that "total nitrogen not including nitrate-nitrogen" only, can be quantitatively determined in the presence of nitrate-nitrogen. With this accomplished it is very probable that

nitrate-nitrogen only can be quantitatively determined by finding the difference between the modified official method and a certain modification of the Kjeldahl-Gunning-Arnold method.

The various data which have been obtained in this investigation seem to warrant the following conclusions:

1. The modified official method (Ranker, '25) is accurate for the determination of total nitrogen in soil extracts and in samples of biological materials, on the plant side.

2. The Kjeldahl-Gunning-Arnold method (Association of Official Agricultural Chemists, '25, p. 8, No. 24) for the determination of "total nitrogen not including nitrate nitrogen" is not accurate in the presence of nitrate-nitrogen. Until this method is perfected, so that it is accurate in the presence of nitrate-nitrogen, it is useless to attempt the determination of nitrate-nitrogen only, by finding the difference between the modified official method and the present Kjeldahl-Gunning-Arnold method.

3. The data obtained do not warrant any alteration in the recommended procedure for the modified official method (Ranker, '25). The stability of the nitrogen complex of certain samples may permit of certain deviations from the recommended procedure, however. This is a matter for individual judgment and will be governed by the particular samples under investigation. These deviations can be made safely, only when suitable qualitative tests are used to detect a loss of nitrogen from the sample.

4. Suitable, accurate qualitative tests for the loss of nitrogen should be as much an integral part of the various quantitative nitrogen methods as the actual quantitative determination itself, whenever it is possible to use them without decreasing the accuracy of the quantitative method involved.

Summary

1. The accuracy of the modified official method for the determination of total nitrogen, as previously reported, is verified.

2. The application of the modified official method is extended to a wide variety of samples, including soil extracts and samples of biological materials, on the plant side.

3. It is demonstrated that the Kjeldahl-Gunning-Arnold method, for the determination of "total nitrogen not including

nitrate-nitrogen," is not accurate in the presence of nitrate-nitrogen.

4. Until the Kjeldahl-Gunning-Arnold method is modified to be accurate in the presence of nitrate-nitrogen, it is useless to attempt the determination of nitrate-nitrogen by finding the difference between this method and the modified official method.

5. Certain details of manipulation and procedure are discussed, and their influence on the accuracy of methods for nitrogen determination is demonstrated.

6. The value of suitable qualitative tests, for loss of nitrogen, to be used as an integral part of quantitative methods for the determination of nitrogen, is demonstrated, discussed, and the use of such tests is recommended.

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SOME CYTOLOGICAL AND PHYSIOLOGICAL STUDIES OF MOSAIC DISEASES AND LEAF VARIEGATIONS[1]

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INTRODUCTION

During recent years, there has been a rising interest in the physiological, anatomical, and cytological study of the mosaic diseases of plants, as a result of the difficulty connected with a determination of the causal agency. Particularly has there been a cytological search of the tissues in an attempt to find either in the living or in the fixed and stained cell an organism which might prove to be associated with the disease. Notable among these studies have been those on tobacco by Iwanowski ('03), Hunger ('05), Delacroix ('06), Dickson ('22), Palm ('22), Goldstein ('24), Rawlins and Johnson ('24, '25), and Eckerson ('26); on potato by Smith ('24); on corn by Kunkel ('21); on the yellow stripe disease of sugar cane by Matz ('19); on the Fiji disease of sugar cane by Lyon ('10), Reinking ('21), and Kunkel ('24); on the mosaic and rosette of wheat by McKinney, Eckerson, and Webb ('23); and on *Hippeastrum equestre* Herb. by Kunkel ('22, '24), and McKinney, Eckerson and Webb ('24); and on *Brassica pekinensis* Skeels by Kunkel ('24).

In the course of these investigations various bodies and cell inclusions of different types have been described. Among other structures found in the cell have been the irregular, amoeboid-like, vacuolate or reticulate bodies which are in many ways comparable to the Negri bodies accompanying rabies, the Guarnieri bodies in small-pox, and the supposed Rickettsia micro-organisms of exanthematic typhus. These amoeboid bodies, as well as the other cell inclusions referred to, have been

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shown to be usually connected with the chlorotic areas in the mosaic-diseased plants in which they occur, and many theories regarding their probable origin and their connection with the disease have been propounded. Hence, the purpose of this investigation was to study both fixed and living cells of numerous types of mosaics in order to determine of how general occurrence the bodies are in the cells of plants affected with mosaic diseases. It was hoped that, through a study of the living cells, some index as to the physiological nature of these bodies might be obtained.

Chlorosis, the pathological condition of which the mosaic disease is a type, has been defined by Clinton ('15) as "that unusual condition in a plant in which the chlorophyll loses its bright green color and becomes yellowish green or even white." The various types of chlorosis have been classified by the same author as follows:

I. Infectious chlorosis.
   A. Communicable through the juice.
   B. Communicable through the tissues.
      a. By buds.
      b. By grafts.
II. Non-infectious chlorosis.
   A. Non-perpetuating.
      a. Affecting plants generally.
      b. Affecting isolated leaves or branches.
   B. Perpetuating.
      a. Through seed.
      b. Through cuttings.
      c. Through buds or grafts.

The mosaic diseases fall naturally in the class of infectious chloroses communicable through the juice. It was, therefore, of interest to make a cytological study of some of the other types of chloroses with the idea of determining how general an associate of chlorosis these various inclusions, and particularly the irregular vacuolate bodies, are. That is, the question arose as to whether these bodies were associated with the loss of chlorophyll in plant cells, or whether they accompanied only the infectious chloroses.

In the majority of the more common horticultural variegated varieties of plants, such as the crotens, oleander, Pittosporum, etc., chlorosis is non-infectious and is not communicable through

either the plant juices or grafting. It is perpetuated in some cases through seeds; in some, through cuttings; and in still others through buds or grafts. However, Baur ('04, '06, '07, '08) showed that there were some variegations, particularly those in the *Malvaceae*, such as *Abutilon Thompsoni*, which were infectious in nature, since in grafting the variegated variety with the green the variegation could be transmitted either from stock to scion or vice versa. There has always been some feeling that Baur's infectious chloroses are not entirely unrelated to the mosaic diseases which are simply infectious chloroses transmitted through the diseased juice. Perhaps, when more is known of Baur's infectious chloroses, it will be found that there is some means other than grafting by which they also may be transmitted.

Histological studies of variegated leaves have been made with the idea of classifying them through their anatomical structure. The outstanding work along this line is that of Funaoka ('24), in which 18 different variegations were studied and classified on the basis of characters of their microscopic anatomy. None of these studies, however, gave any indication of the presence in the cells of bodies and inclusions comparable to those found in certain of the mosaic diseases. Hence, a cytological study of representatives of the various types of variegations was planned in order to determine whether there are any inclusions in the chlorotic areas comparable to those in the chlorotic areas of the mosaic diseases studied, and if so, whether they are in any way similar. It was hoped that such a study would give some indication of a possible relation between the infectious and non-infectious variegations and the mosaic diseases which are due to, and transmitted by, the filterable virus in the diseased juice.

Discussion of Literature

A mosaic disease was first carefully studied by Mayer ('86) in tobacco, but although he found that it could be transmitted by inoculation of the mosaic juice into healthy plants and that it was inactivated by heating to 80° C., he made no cytological study of the tissue. He did examine the sap but found only starch grains and calcium oxalate crystals. Koning ('99) drew

the cross-sections of a few leaves but failed to observe any pathological inclusions. It was Iwanowski ('03) who first made a detailed cytological study of the leaves of infected plants. He studied living cells as well as cells of tissues which had been fixed in Flemming's solution, in osmic acid, and in boiling absolute alcohol, and of these he found the latter the most successful. In the living cells he observed a greater abundance of oxalate crystals in the chlorotic than in the green area; clear plate-like crystals which he described as being similar to waxy material, but less refractive; cells with granular bacteria-like inclusions which, however, never form uninterrupted complexes; and finally, plasma-like accumulations reminding one of parasitic amoebae. In the material fixed in boiling absolute alcohol and stained with methylene blue and eosin he found structures which he thought were zooglea of bacteria with approximately the same form as those of the plate-like crystals in the living cells. Also, he observed, as in the living cells, that the plasma accumulations took the eosin more deeply than did the cytoplasm, and these were most frequently found in the neighborhood of the nucleus or the crystals.

To these observations he gave the following interpretations: the plate-like crystals in the living material gave rise to the striate zooglea in fixed material, the latter being composed of organisms which were the causal agency of the mosaic and were small enough to pass through a filter; the amoeboid bodies, he believed, were the result of the reaction of the cell to the irritation of the causal agency.

Hunger ('05), in checking Iwanowski's work, showed that the so-called mosaic disease bacteria of Iwanowski, as well as the zooglea, disappeared when the cell was treated with phenol chlor-hydrate, while the other cell-structures remained the same. That is, he obtained a solution of the plate-like crystals with phenol chlor-hydrate.

Delacroix ('06), in following the method of Iwanowski of fixing the tissues in boiling absolute alcohol, was able to observe the amorphous bodies and the zooglea. However, when fixed in cold absolute alcohol he obtained no such structures. From these observations he concluded that they were deposits of

substances which were dissolved in the cell sap and precipitated by the boiling alcohol. He apparently did not account for the fact that they also appeared in the living cells. In view of this latter fact his results could no doubt be interpreted as having been brought about by the solution of the bodies in cold but not in hot absolute alcohol.

Lyon ('10), in studying the Fiji disease of sugar cane, found one or more plasmic bodies in every cell of the abnormal tissue. They were generally rounded in contour although they might be variously lobed or distorted at times, and their constituent substance was much denser optically than that of the cytoplasm of the cane, making them readily visible even though all the contents of the cells were clear and colorless. They were devoid of cell walls or other precipitated secretions. In interpreting these observations, he concluded that "In these foreign bodies we recognize an interesting and dangerous parasite quite new to the science of sugar cane pathology. It belongs to a very small group of lowly organisms whose position in the realm of living things is a subject of dispute among naturalists." Apparently he considered that they were plasmodia of a myxomycete, similar to *Plasmodiophora Brassicae*, which break up to form spores, the latter being freed with the disintegration of the tissue. Infection, then, according to him takes place by the entrance of the swarm spores through the roots and up through the vessels to the leaves.

Matz ('19), in his studies on the yellow stripe disease of sugar cane, observed granular, plasmodium-like substances in the yellow-striped cane leaf and stem tissues, and found the mass made up of small, hyaline bodies, the entire mass being in the form of a compact plasma with the bodies less than 1 μ in length. They seemed, however, less clearly defined than masses of bacteria. Later, in 1922, he considered them as co-generic with *Strongyloplasma Iwanowski* Palm, described by Palm in the chlorotic areas of the tobacco leaves infected with mosaic.

The term *Strongyloplasma Iwanowskii* was applied by Palm ('22) to the very small granules (at the most .5 μ in length) which he observed in the chlorotic areas of tobacco leaves infected with the mosaic disease. He found them staining black with haematoxylin, and frequently forming irregular agglomera-

tions which sometimes filled the entire cell lumen. These very small bodies he believed agreed in every respect with the *Strongyloplasma* of von Prowazek and Lipschütz. He also observed the fairly large corpuscles usually in contact with, or in close proximity to, the nucleus, staining gray with haematoxylin and very light red with eosin. In the living cells the latter were present and were denser and more opaque than the surrounding cell plasm. Although they were displaced at times by the streaming of the protoplasm, he saw no evidence of any automotive movement. In the interpretation of his results he agreed with Iwanowski ('03) that these latter bodies were the product of the reaction of the virus carrier on the cell plasm, and believed that they were homologous with the Guarnieri bodies. The very small granules were, according to him, the causal agency, and he therefore concluded that in the mosaic disease of tobacco we are dealing with a disease which belongs etiologically to the chlamydozoonoses.

The presence of the plasmodial bodies in the Fiji disease of sugar cane as described by Lyon ('20) was verified by Reinking ('21), who reported finding the bodies as light-colored in the younger galls and brown and granular in the older ones. He found them in the young shoots arising from the base of diseased plants, in the rotted roots, and in the base of the stem. He concluded that, although the presence of the bodies throughout the plant has not yet been demonstrated, their presence would indicate that "the fungus is responsible for the disease."

Kunkel ('24) studied the bodies in connection with the Fiji disease in great detail and found particularly in the young galls that they were frequently small and composed of a deeply staining granular mass with occasional vacuoles. In many cases, they possessed short blunt appendages which were more hyaline than the main part of the body and resembled pseudopodia of amoebae. In maturing and old galls, the bodies became larger and less dense, frequently containing large, deep-staining granules in a vacuole surrounded by a definite membrane. He frequently found two bodies, one in either end of the cell, in the case of dividing cells, thus indicating that these plasmodial bodies do possess the power of division and of growth. These facts,

together with the distribution of the bodies in the gall tissues, their apparent manner of spreading, and constant association with the earlier stages of gall formation, indicate, he believed, that they belonged to a parasitic organism. However, he still considered it an open question as to whether they were inert cell inclusions resulting from some physiological disturbance, or represented a new type of plant parasite.

Kunkel ('21) has also studied the mosaic disease of corn, and compared the bodies found there with those in the yellow stripe disease and the Fiji disease of sugar cane. Studies were made both of living and fixed material, and in all chlorotic areas irregular amoeboid bodies were found in association with the host cell nucleus and frequently appeared to be attached to it. They showed a structure similar to that of protoplasm but somewhat more dense and opaque. In the living cells, he never found them showing any automotive movements or change of form. Frequently they appeared naked, but at times a thin limiting membrane could be observed. Vacuoles were present in some and in others they were absent. Also, there were in some of the bodies numerous dark-staining granules which tended to be angular rather than spherical and never showed any semblance to nuclei. Those cells possessing the bodies were found to increase in size and to show nuclei which also were larger than normal. During the early stages of the disease the bodies were very minute and apparently increased in size with the progress of the disease. The outstanding characteristics, then, as he saw them, were that they appeared to grow, that they showed a structure like that of protoplasm, that they stained like protoplasm, and that they tended to be amoeboid in shape. Also, the tendency to cluster around the nucleus was comparable to the similar tendency among other known intracellular parasites such as the swarm spores of *Chrysophlyctis endobiotica* Schilb in the potato wart disease (Orton and Kern, '19). The bodies, therefore, in his estimation, had many of the characteristics of a living organism. However, they had never been cultured, and until that might be possible they could not be definitely considered as the causal agency.

The vacuolate bodies found here were entirely different from

the granular mass described by Matz ('19) in the yellow stripe disease of cane, since the latter did not stain like protoplasm, did not show a protoplasmic structure, were not vacuolate, did not contain any of the dark-staining granules, and were not plastic. Neither were they like the bodies associated with the Fiji disease of cane, nor like the plasmodia of *Plasmodiophora Brassicae*. They did, however, resemble remarkably the Negri bodies associated with rabies in the brain cells of the diseased dog.

Similar vacuolate bodies have been found in wheat plants infected with the rosette disease and the mosaic-like leaf mottling by McKinney, Eckerson, and Webb ('23a). They were found in the roots, crown tissue, leaf sheaths, and leaves, never necessarily occurring in close contact with the nucleus. Their contents were rather homogeneous in structure, and they contained many large and small vacuoles in which granules could be observed showing Brownian movement. No independent movement was ever observed in the living cells. From these observations they concluded that, although the bodies might be a stage of some definite parasite, yet their distribution in the host tissue and their apparent parallel development with that of the host cells did not seem to conform exactly with the distribution and development of any plant parasite known.

The same investigators (McKinney, Eckerson, and Webb, '23) have described similar bodies in the chlorotic areas of the leaves of *Hippeastrum Johnsonii* which were infected with the mosaic disease, as did also Kunkel ('24a) in *Hippeastrum equestre* Herb. Kunkel found them to be similar to those in corn as regards distribution, although the *Hippeastrum* bodies were considerably smaller. They were never found in tissues until the mosaic blotching appeared, and the size of the bodies was directly proportional to the degree of chlorosis, the lighter areas showing the largest bodies. Kunkel also found such amoeboid-like structures in the chlorotic areas of mosaic-diseased *Brassica pekinensis* (Skeels), which were similar to those in corn and in *Hippeastrum equestre* Herb. both in structure and in staining reactions. The *Brassica* bodies differed, however, in not being primarily associated with the nucleus. He then concluded, from all of his observations on the bodies in the Fiji disease of

sugar cane, in the mosaic disease of corn, in *Hippeastrum equestre* Herb., and in *Brassica pekinensis* (Skeels), that the amoeboid bodies associated with these diseases might represent only one stage in the life history of the causal organism, and that at another stage they might be so small and plastic that they could pass through the fine pores of a filter and escape detection under the microscope. If this were the case, they would probably become visible only after a certain period of growth within the cell of the host. For the time, however, he added, "we must be content with the knowledge that intracellular amoeboid organisms accompany the mosaic disease in several plants, that these bodies look like living organisms, and that in corn and *Hippeastrum* they are associated with chlorosis in such a way as to account for the mosaic pattern in the leaves."

Smith ('24) studied the leaf and stem tissue of varying age from mosaic-diseased potato plants and found in the chlorotic areas similar vacuolate bodies which showed definite walls and bore, as he said, "a superficial resemblance to some kind of protozoal organism." They were usually in close association with the nucleus of the host cell, and in the living cells they failed to show any automotive movement. In view of these facts and the further fact that in the light green areas in which these bodies were found the general disintegration of the tissue seemed to be considerable, he came to the conclusion that they were some type of degeneration product of the cell and probably of the nucleus, induced by the mosaic, and that they were the effects rather than the cause of the disease.

Contemporaneous with the studies on the mosaic disease and the associated amoeboid-like bodies in corn, sugar cane, potato, *Hippeastrum*, and *Brassica*, have been several notable contributions to a study of similar structures in the chlorotic areas of the mosaic disease in tobacco, tomato, and related genera. Dickson ('22), in observing living free-hand sections of the tobacco leaf, found in the chlorotic areas of leaves in the advanced stages of the disease, among the vacuolate bodies and the plate-like crystals, numerous smaller bodies exhibiting an erratic movement. He considered them as flagellates but in spite of careful staining he could obtain no proof of this. In fixed material he

found minute dark-staining bodies, .3 μ long and slightly less in width. They were found particularly in the border parenchyma of the vascular tissue of diseased leaves, but were also observed in close contact with the walls of the chlorenchyma cells and, in some cases, surrounding the chloroplasts. The vacuolate bodies, comparable to those described by Kunkel in corn, he believed were not the causal agency, but were, on the other hand, secondary in nature. He attempted to culture these very small bodies but was unsuccessful in isolating them from the virus. The plate-like crystals which have been described in connection with the mosaic disease of tobacco were believed by him to be due to a product of chlorophyll degeneration combined with changed plastid protoplasm, in view of the fact that Ewart had shown that CO_2, combined with chlorophyll in the presence of water to form xanthophyll and a colorless waxy substance,

In connection with these studies Dickson also made an anatomical study of numerous mosaic-diseased plants, from which he drew the following conclusions regarding the anatomical characteristics of the mosaic disease in general:

1. There was a difference in thickness between the chlorotic and the green area, the ratio being about 2:3, due to hypoplasia of both palisade and spongy parenchyma cells in the chlorotic areas.
2. The dark green areas exhibited hyperplasia.
3. There was a regular arrangement of cells in the light area, thus reducing the intercellular spaces.
4. The epidermal cells were smaller in area but deeper over the hypoplastic areas than over the normal.
5. Hypoplasia was accompanied by a degeneration of cell contents.
6. Disintegration of the chloroplasts was accompanied by the appearance of the very small hyaline bodies in rapid movement, the semi-crystalline plates believed to arise from degenerate chloroplasts, and the vacuolate bodies.

The vacuolate bodies in the hair cells of the mosaic-diseased tobacco were studied by Goldstein ('24) in the living condition. She found that in the chlorotic areas the hair cells and epidermal cells contained the vacuolate, more or less amoeboid bodies, and the crystals, both of which have been described and illustrated many times since their first description by Iwanowski ('03). However, she watched them and studied them more intensively

in the living condition than had been done heretofore. She found that the bodies bore no definite relation to the nucleus, but were simply in the cytoplasm and carried about in it when the protoplasm was streaming actively. Not only did she see the body carried around the cell in the protoplasmic streams but also she noticed an apparent change in form which might be described as an indication of automotive movement. In the pseudopodia of the more active bodies she occasionally observed a hyaline ectoplasmic-like cap bordered by a membrane. She interpreted her observations of such membranes as convincing evidence in favor of the view that the bodies are surrounded by a definite plasmatic membrane. Also, she found that treatment with acid caused the contents of the bodies to shrink, leaving visible the definite limiting membrane.

She mentioned the crystals but gave no results of her work on them, promising a paper in the near future. She did, however, treat the cells with various fixing fluids, watched the influence of these under the microscope, and found that the crystals lost their typical form, becoming long, irregularly lobed and striated masses which were stained deep yellow by the fixatives. This accounted for the appearance of the striate masses in the fixed mosaic tobacco tissues. She concluded from these observations that it might be possible that such plastic bodies as these would be able to pass through cell walls and the pores of bacterial filters just as the nuclei were observed to migrate from cell to cell in *Tradescantia* (Miehe, '01), thus explaining the nature of the virus.

Rawlins and Johnson ('24, '25) have studied cytologically the fixed tissue of the mosaic tobacco leaves, and have described three types of cellular inclusions,—the yellow-staining striate material radiating from the nucleus, small black-staining bodies, and vacuolate bodies varying in size from those just visible to those slightly larger than the nucleus. They found that the development of these inclusions was inhibited by temperatures which inhibited the expression of the mosaic. Also, it was observed that only 20 per cent of the plants showing the symptoms out of doors showed the bodies indoors, whereas 80 per cent of those in the greenhouse showed them. They have

attempted to show the sequence of the appearance of these various types of bodies. The first to appear was the striate material, the small dark-staining type, and the crescent-shaped vacuolate type, and they felt that the small bodies were a stage in the development of the vacuolate type. The crescent-shaped type definitely gave rise to the rounded vacuolate type which then persisted along with the striate material throughout the life of the leaf. They considered that the amorphous nature of the striate material indicated that it was a product of the diseased cell or of the causal agency.

Very recently Eckerson ('26) has described what she considered an organism of the tomato mosaic. She studied the tissues of tomato plants at various short intervals after inoculating the healthy plants and was able to observe at 24 hours after inoculation flagellate organisms in the veins and adjacent tissue, and the chloroplasts near the veins showed signs of dissolution. As the time after inoculation increased, the bodies became more numerous and the dissolution of the chloroplasts continued, while the bodies within them increased in size. Seven days after inoculation many of the chloroplasts in the palisade layer were in the process of liquefaction, while the remaining plastids contained non-motile bodies which seemed to be early stages of spore formation. Ten days after inoculation, when the leaflets began to show mottling, the palisade cells were partially disorganized, the cytoplasm was gone, the chloroplasts partially dissolved, and the remaining ones contained spores. These disorganized cells were usually bounded by groups of cells in apparently perfectly healthy condition. She has included many illustrations of both the spore and flagellate form, showing the nature of the organism. It is interesting that the organism should have been found associated with the chlorotic areas of mosaic-diseased plants, but she has not yet demonstrated that it is the causal agency by isolating it and inoculating the resultant culture into healthy plants. Moreover, it must be remembered that the size of the particle of the causal agency has been determined by Duggar and Armstrong ('23) through filtration experiments to be approximately the size of the particles in a fresh 1 per cent solution of haemoglobin, which is 30 μμ. The smallest size which Eckerson

gave for the flagellate forms she described is 2–4 μ, and this is approximately 1000 times the size of the particle as determined by Duggar and Armstrong.

The work of Nelson ('23) has not been included in this discussion, since it has been completely refuted by other investigators who have shown that the protozoan-like bodies which he described occur normally in the phloem tubes of healthy plants.

Structures similar to Nelson's bodies have been described by Klebahn ('26) in the sieve tubes of *Anemone nemorosa*, and he believes that evidence is strongly indicative of their being the cause of the disease called by him "alloiophylly." He considers that these bodies are closely related to the bacteria in that they show no definitely organized nucleus, and that their size seems to fall within the limits of the size of bacteria; and he applies the term *Scolecosoma anemones* to them. They have not, however, been found to reproduce by simple fission, and for this reason as well as the fact that they exhibit a great variation in form, they differ from the bacteria. He has, therefore, concluded that they are a "neuen Organismengruppe die etwa zwischen Bacterien und Flagellaten vermittelt." According to him, it is possible that his *Scolecosoma* and Nelson's bodies belong to the same or nearly related species, and it should still be an open question as to whether or not these belong to the same group of organisms as do the vacuolate bodies described as accompanying many of the mosaic diseases.

There have also been numerous cytological and physiological studies made on the chloroses of many of our variegated horticultural varieties. Although anatomical studies have been made, particularly by Funaoka ('24), there have never been found inclusions of the nature of those described as occurring in several of the mosaic-infected plants. However, the investigations have led to results so closely parallel to those obtained from work on the mosaic plants that a resumé of them would be of interest here in showing the possible connection between the two types of chloroses.

Masters ('69) considered albinism as a change due to the deficient formation of green coloring matter or chlorophyll. He distinguished between this condition and etiolation by the

fact that in the former chlorophyll seemed never to be formed in the affected parts, even if they were exposed to light, while an etiolated organ placed under favorable circumstances speedily assumed a green color. Later, Weiss ('78) explained variegations on the assumption that white spots were caused by the presence of air which was held in the intercellular spaces under the epidermis. He showed that leaves which were exhausted of air under water, by means of a pneumatic pump, lost the white spots. However, Dalitsch ('86) contributed the first accurate observations on the cause of variegation. He defined the white spots as due to chlorophyll-free cells in the fundamental tissue.

Saposchnikoff ('89) studied the starch content of chlorotic and green areas of variegated leaves and found starch only where chlorophyll was present. When, however, the leaves were placed in sugar solution, starch was found in equal amounts in the chlorotic and the green areas. In this connection, Winkler ('98), from a study of leucoplasts, chromoplasts, and chloroplasts, concluded that, whether the stroma was or was not stratified, whether it contained chlorophyll or some other pigment, whether the pigment was granular or crystalline, whether the plastid was large, small, distorted, or smooth, the stroma was always, when not too greatly reduced, able to form starch, if sugar were present.

Pantanelli ('05) made a physiological study of the variegations and found that it was possible to explain them on the basis of enzyme action. He found that the chlorotic areas were characterized by a decrease in chlorophyll content, increase in the accumulation of oxidizing enzymes, an increase in the osmotic pressure, lack of accumulation of mineral and organic salts and sugars, and a limitation of growth processes. His explanation of these observations is given in the following theory regarding the etiology of variegations. The first indication was probably an abnormal accumulation of the oxidizing enzymes, which disturbance probably took place in the stem or root; and then the disturbing material was carried up by material transport through the sieve tubes to the various parts of the plant. In the chlorenchymatous cells, it led to a destruction of the chlorophyll and to a general disease of the protoplasmic parts, which was evidenced by an increase in turgor. Further investigation

showed that the protoplasm and the plastids were disorganized and digested by the enzymes which were developed in abnormal quantities. The observed increase in osmotic pressure, he believed, was probably due to the increase in disintegration products of smaller molecular dimensions. He therefore considered that the pattern of the variegation followed the veins, through which the agency was carried and from which it was frequently distributed on one side only, making the vein the boundary between the green and the chlorotic areas.

Baur ('04) distinguished between the non-infectious and the infectious types of variegation, the former being transmitted through the seed, whereas the latter, although they could not be transmitted through the seed, could be passed on to healthy plants by grafting a variegated twig on a healthy stock. He found infectious variegation to be quite frequent among the *Malvaceae*, and he investigated in great detail the variegated variety *Abutilon Thompsoni*. A microscopic study of the leaf tissue revealed nothing of the nature of a causal organism, but only a reduction in the size and number of plastids and in the amount of chlorophyll contained by these in the chlorotic areas. In an attempt to determine the nature of the virus he tried many methods of transmitting it to healthy plants, but was successful only in the grafting experiments. In 1906, he concluded that the virus was not an organism but highly organized products of metabolism, which in a certain sense, possessed the power of growth. Such products passed through the cells of the plant, and in the embryonic cells of the young leaves they found free side-chains to which they attached themselves. In these cells, then, it was believed that the toxin was again formed anew. This physiological explanation is quite analogous to a similar explanation given by Hunger ('05) as the cause of the mosaic disease of tobacco.

A very similar theory was sponsored by Molisch ('08) in connection with his studies on *Abutilon Thompsoni*. He studied both living and stained material and found no structures which might be interpreted as living organisms. He also tried cultivating the virus on artificial media and on an extract of *Abutilon* leaves, but was entirely unsuccessful.

At this time, Kränzlin ('08) made a study of the pigments connected with variegated plants, and he obtained results which were confirmed later by the work of Colon ('19) in a study of the pigments associated with the mosaic diseases. These results indicate that the same pigments are present in both the chlorotic and the green areas, the difference being primarily one of quantity of pigment. In the chlorotic areas he found less of each of the normal leaf pigments, that is, he found a similar decrease in chlorophyll and in carotin. Colon, in his work, obtained similar absorption spectra from the two areas and concluded that the chlorotic nature of the spots in the yellow stripe disease of cane was not primarily due to a decomposition of the chlorophyll as such.

Küster ('19) found the veins incompletely developed in the chlorotic areas of the marginally variegated leaves of *Acer platanoides*, and hence concluded that degeneration or incomplete development of the green chloroplasts was probably caused by diminished nourishment. This was, however, refuted by Funaoka ('24), who studied the relative frequency of the veins in the chlorotic and the green areas of 14 species. The results showed that in 9 of the species there was an equal frequency, in 3 species there was a thicker network of veins in the chlorotic than in the green areas, whereas in a single species, *Euphorbia marginata*, the net was thicker in the green areas. In *Richardia Elliottiana* the vascular network was not developed in the chlorotic area. Hence, from his observations he concluded that in many plants the cause of variegation could not be traced back to an insufficient supply of vascular bundles and a resulting poor nutrition.

Funaoka also made an extensive study of 18 different variegations, and from his observations was able to classify them on the basis of their microscopic anatomical characters. The paper was well illustrated with semi-diagrammatic drawings showing that the chloroses might be due to a loss of chloroplasts in one or more layers of cells in the leaf (periclinal variegations), or to an anticlinal division of the green and white areas of the mesophyll, or a lack of differentiation of one or more layers, particularly the palisade layers. No indication of any structure resembling a microorganism was described.

From an investigation particularly of variegated varieties of *Zebrina*, Tsinen ('24) concluded that from the cytological point of view variegations occurred as the result of an alteration in the plastid mechanism of the cell. This alteration could take place before the differentiation of the plastids from the chondriosomes, at any stage during their development, or after they were mature, thus explaining the various types of variegations found.

Materials and Methods

Living and fixed materials were studied in plants infected with the mosaic disease and in the variegated plants. For a study of the living material the following vital stains were tried, —methylene blue, neutral red, bismarck brown, dahlia, and brilliant cresyl blue, the most favorable being the latter in concentrations of 1:10,000 and less. Thin sections of even the thinnest leaves, such as those of *Bougainvillea*, could be easily made by holding several pieces of the leaf within the same piece of pith, and then making free-hand sections of them. The sections were mounted in water and studied without any stain, after which a drop of brilliant cresyl blue solution, 1:10,000 was drawn under the cover-slip. The large vacuolate bodies, particularly those in *Petunia*, were found to stain very well after an exposure to the dye of from fifteen minutes to several hours.

For the fixed and stained materials, the following fixatives were tried,—Bouin's fluid (see Lee, '13, p. 65), Flemming's weak solution (see Chamberlain, '24, p. 25), medium chromacetic acid (see Chamberlain, '24, p. 25), osmic-sublimate mixture (see Lee, '13, p. 50), and an acetic-alcohol-formalin mixture (see Rawlins and Johnson, '25). In all cases small pieces of tissue were taken in order to insure rapid penetration.

These 5 different fixatives were tried on the tissue of tobacco, poke, and geranium mosaics, and on the variegations in *Evonymus japonica* Linn., *Ficus Parcellii* Veitch, *Ligustrum ovalifolium* Hassk., and *Abutilon pictum* Walp. in combination with each of the following stains,—Flemming's triple stain (see Chamberlain, '24, pp. 59–62), Delafield's haemotoxylin (see Chamberlain, '24,

pp. 45–48), and Haidenhain's iron alum haemotoxylin (see Chamberlain, '24, pp. 41–45).

The best results were quite generally obtained when the chrom-acetic acid was used as a fixative and was followed by the Haidenhain's iron alum haematoxylin. Counterstaining with the Orange G was found to be very desirable, since the vacuolate bodies seem to show a strong affinity for it. The most satisfactory method for introducing the counterstain was to dilute a 1 per cent solution of the stain in clove oil to a light amber color and place the slides in it for 10 to 15 seconds before placing them in xylol. This combination of chrom-acetic acid as a fixative, and Haidenhain's iron alum haematoxylin counterstained with Orange G was then used in all subsequent preparations, since it seemed the most generally successful.

With this technique, then, the following mosaic diseases were studied,—tobacco (*Nicotiana Tabacum* Linn.), petunia (*Petunia* sp.), columbine (*Aquilegia caerulea* James), pokeweed (*Phytolacca decandra* Linn.), and Jimson weed (*Datura Stramonium* Linn.). As to variegations, the following were chosen because they represent both Monocotyledons and Dicotyledons, because there are among them both infectious and non-infectious chloroses, and because they show quite different and distinct anatomical variations: *Homalomena cordata* Schott, *Ficus Parcellii* Veitch, *Nerium Oleander* Linn., *Coleus Blumei* Benth. var. "Mrs. Kirkpatrick," *Bougainvillea glabra* Choisy var. "*variegata*," *Pittosporum Tobira* Ait. var. "*variegatum*," *Evonymus japonica* Linn. var. *argenteo-variegata*, and *E. japonica* Linn. var. "*mediopicta*" Hort.

The cytological studies were made with a Zeiss microscope equipped with a 1/12a fluorite or semi-apochromatic objective, and a 4-mm. achromatic objective number 6. While studying the preparations the binocular tube was used with number 4 oculars, but all drawings, with the exception of text-figs. 2, 3, and 4, were made with the monocular tube, using a 12x compensating ocular. The text figures just mentioned were drawn with a 2x ocular and the 4-mm. objective. All drawings were made with the aid of a Spencer camera lucida.

Observations and Discussion

Mosaics

Preliminary to the following work, a survey was made of living and fixed material of many of the mosaics at the author's disposal, with the idea of determining those which seemed most favorable for more intensive study. Several which are not mentioned here, such as *Crotalaria*, geranium, and poinsettia, have not as yet been sufficiently studied to warrant a report in the present paper, but it is hoped that the work on these may be forthcoming in the future. The studies given here have been made upon tobacco, *Petunia*, *Datura*, pokeweed (*Phytolacca decandra*), and *Aquilegia caerulea*.

1. Tobacco.—The work began with a study of the hair cells in the chlorotic areas of tobacco, since the inclusions here have been frequently described and since these cells seemed to offer such good material for the study of the bodies and crystals in living cells. Epidermal and hair cells were studied in the living condition, while in the case of fixed materials cross-sections of the leaves were used. In the living cells, irregular, vacuolate, amoeboid-like bodies together with clear plate-like crystals were found just as illustrated by Goldstein ('24). No indication of anything comparable to a nucleate structure was observed in either the fixed or living bodies, and they appeared to lack a limiting membrane of any sort. In the living cells, when protoplasmic streaming was sufficiently rapid both the bodies and the polygonal, flat, plate-like crystals were carried with it around the cell, but at no time could any movements which might be interpreted as automotive be discerned. The vacuolate bodies apparently changed their form somewhat, but all of these changes could be explained by the fact that the body was being turned over in the stream just as were the large crystals. There seemed to be no connection between the nucleus and the vacuolate bodies, the only times when they were adjacent to each other being when the nucleus acted as an impediment to the body as the latter was being carried through the cell in the protoplasmic stream. Both the bodies and the crystals were strikingly similar to those in *Petunia*, and since they will be described later, it is not necessary to go into detail at this point.

Rawlins and Johnson ('25) showed that in tobacco mosaic the bodies were found in 80 per cent of the mosaic plants grown under greenhouse conditions, whereas only 20 per cent of those grown outside exhibited them. In the present investigation it was also found that in the tobacco the inclusions were of more common occurrence in the greenhouse plants than in those grown out of doors. The idea arose that perhaps the ultra-violet rays which reach the plant when it is grown outside but which are cut out by the glass of the greenhouse might be, at least in part, the cause of this difference. With this idea in mind the following experiments were conducted.

EFFECT OF THE LONGER ULTRA-VIOLET RAYS ON TOBACCO PLANTS INFECTED WITH MOSAIC DISEASE

Ultra-violet rays may be divided into two diametrically opposed categories in regard to the action on living organisms. Those with the longer wave-lengths, 400–290 μμ (4900 A. U.–2900 A. U.) are commonly known as the biological rays. Since they are relatively penetrating, they include that range of the ultra-violet which may be present in the solar spectrum as it reaches the earth's surface. As to their action on living organisms, they are characterized by being chemically oxidizing and hence metabolic synergists. Opposed to this division, are those with the shorter wave-lengths which are commonly termed abiotic rays, because of their action on living protoplasm. In contrast with the longer rays, they are chemically reducing and metabolic depressors. They are so readily absorbed that penetration is very slight, hence they are never present in the solar spectrum as it reaches the earth's surface. In fact, the shortest wave-lengths obtained in the solar spectrum are about 291 μμ. All the shorter wave-lengths are absorbed by the earth's atmosphere. Because of the poor powers of penetration the abiotic rays are known to be superficial in action, being unable to penetrate the human epidermis. It is, then, recognized that the abiotic rays are lethal to bacteria and other living organisms, and these are concerned in sterilization processes.

Since the abiotic rays are so poorly penetrating and since it is the biological rays which have such profound reactions on the tissues of higher animals, it was the effect of these latter

rays on the tobacco plants infected with mosaic in which the author was primarily interested. In the experiments 2 types of lamps were used,—the Alpine Lamp, of the Hanovia Chemical Co., and the Air-cooled Quartz Mercury Vapor Lamp, of the Burdick Cabinet Co. Both the biological and the abiotic rays can be obtained from these lamps, depending on the distance between the burner and the object exposed. A column of air of 36–40 inches will absorb the abiotic rays, leaving only the longer biological rays. Therefore, to test the effect of the abiotic rays, the object is placed within 6 inches of the burner, whereas in an investigation of the longer wave-lengths, the object is placed at least 36–40 inches from the burner.

In the literature there has been very little experimental work on the effect of the biological ultra-violet rays on the tissues of plants, hence many difficulties arose in connection with the details of applying the lamp. The plants were found to burn most easily, particularly with the new Burdick lamp, so the time of exposure and the working distance (i. e., the distance between the burner and the plant) had to be determined for each lamp.

The best results were obtained with the Hanovia lamp, because it contained a very old burner in which the intensity of the rays had been decreased considerably. With this lamp it was found that the plants could be given a treatment of 30 minutes at 40 inches daily without bringing about fatal injuries to the plants, although they did become severely dwarfed.

Six plants were inoculated with filtered juice from the leaves of mosaic-infected tobacco plants, and the ultra-violet treatment was begun 2 days later, after the plants had recovered from the ill effects of the inoculations. They were rayed daily for 30 minutes at 40 inches, being kept, during the remainder of the day, under normal greenhouse conditions. At the end of 9 days all 6 plants showed the normal mosaic symptoms. With continued treatments the plants gradually became more and more dwarfed, and with the noticeable dwarfing the symptoms became less evident. At 20 days after inoculation the plants had apparently completely lost all of the typical mosaic symptoms. The treatment was continued for an additional 8 days, thus making the entire treatment 4 weeks in duration.

After treatments were stopped the plants were kept under observation in the greenhouse for 40 days, at the end of which time 2 of the 6 plants had re-developed the mosaic symptoms, 2 remained uniformly green, and the remaining 2 succumbed to the treatment which they had received. Therefore, the loss of symptoms after 18 days of exposure to the ultra-violet was apparently simply a masking of them, such as has been observed in plants which have been kept under blue light (Lodewijks, '10; Chapman, '17; and Dickson, '22). There was not a permanent curative effect. As has been noted, the plants were dwarfed, showing that the conditions to which they were subjected during treatment were not optimum for growth, and it is a well-known fact that the symptoms are the most prominent when the plants are growing rapidly. Therefore, it was not surprising that the disease was masked.

This masking of the mosaic symptoms in tobacco plants which were exposed to the biological ultra-violet rays would seem to substantiate the work of MacMillan ('23), in which he observed a masking of the mosaic symptoms in potato plants grown at high altitudes. He suggested at that time that the masking might be due to the biological ultra-violet rays which are more abundant at high than at low altitudes, and which, he believed, stimulated chlorophyll production in these cells which would be chlorotic under ordinary circumstances.

Pieces of the leaves were taken at random from the plants when the treatment was completed, and studied in fixed material as well as in the living condition. The vacuolate bodies and the crystals were still present in some of the hair cells, indicating that the ultra-violet rays were not the cause of the absence of the bodies in the mosaic plants which were grown out of doors. Little effect was found in the tissues other than a more uniform distribution of the plastids and a more constant differentiation of palisade and spongy mesophyll throughout the leaf of the rayed mosaic plants; likewise, in the latter there was an increase in the number of epidermal hairs over that in the control plants.

Experiments conducted in the same manner with the Burdick lamp gave comparable results, but in the case of this new burner the intensity of the rays was so great that the burning and

injury to the plants were difficult to avoid, even with exposures of only 1 or 2 minutes at a working distance of 50 inches. This again illustrates the great difference between burners and shows that the time and working distance of exposure must be determined for each burner with each type of plant. From these results it can be concluded that, in the case of the tobacco plants infected with mosaic, the only effect of the biological ultra-violet rays is the inhibition of normal growth, which in turn causes a masking of the mosaic symptoms. Moreover, the fewer inclusions in the cells of the plants grown out of doors than in the greenhouse plants cannot be explained as a result of the action of the biological rays of ultra-violet light.

EFFECT OF THE SHORTER ULTRA-VIOLET RAYS ON THE MOSAIC VIRUS

This study of the effect of the biological ultra-violet rays on the tissues of plants infected with mosaic suggested the idea of determining the effect of the abiotic rays on the virus itself. As has been explained, the shorter wave lengths are not penetrating and can be obtained only at the very short working distance of 6 inches. Hence, the effect of the abiotic rays on the virus cannot be studied by subjecting the plants directly to these for two reasons: first, the shorter wave-lengths are so poorly penetrating that they cannot pass through the epidermal cells; and, second, the plants so treated would be immediately burned and killed. In these studies, therefore, the effect of the rays on the virulence of the mosaic tobacco juice was investigated.

Fresh mosaic tobacco leaves were ground, the juice filtered through cotton, water added to make a 1 : 4 dilution, and this filtered through a spherical atmometer cup. This procedure removed from the juice the chlorophyll, which, if it had been present during the subsequent treatment, would probably have absorbed all of the shorter wave-lengths and a determination of their effect on the virus would have been impossible. The filtered juice, however, was a clear solution, thus avoiding the suggested difficulty.

Five-cc. portions of this clear, filtered mosaic juice were placed in uncovered 50-mm. petri dishes and then exposed to the ultra-violet rays at a distance of 6 inches for varying periods of time as

seemed desirable from the preliminary experiments. Ten plants were then inoculated with the rayed juice in the case of each exposure, and, as a control, 10 plants were inoculated with the filtered juice which had not been rayed. The experiments were run at 2 different times during the winter and in different compartments of the greenhouse, with similar results, thereby assuring that environmental factors were neither favoring nor masking the expression of the symptoms. The virulence of the virus was considered to be indicated by the percentage of plants showing the mosaic symptoms. Since preliminary experiments showed that the virus was in no way inactivated by an exposure of 5 minutes, exposures shorter than that were not repeated in these two series of experiments. In this work, the new Burdick lamp was used, operating at 8 amperes and 70 volts. The results are given in the following tables.

TABLE I

INFLUENCE OF ABIOTIC RAYS ON THE MOSAIC VIRUS

Length of Exposure	Results	
	1st series	2nd series
Control	9 affected in 9 days	10 affected in 11 days
5 minutes	8 affected in 9 days	10 affected in 12 days
10 minutes	5 affected in 9 days	6 affected in 14 days
20 minutes	1 affected in 9 days	2 affected in 14 days
30 minutes	None affected in 9 days	1 affected in 14 days

In the experiments the remainder of the plants continued healthy for a period of 5 weeks and were discarded at that time. These results show clearly that the virus has, with sufficient exposure to the abiotic rays, been permanently inactivated. The same data show that the process of inactivation has been gradual, since there is some reduction in the virulence of the virus with an exposure of 10 minutes. The exposure is not lethal, however, until the virus has been subjected to the rays for a period of 30 minutes. The single plant which succumbed to the disease in the exposure of 30 minutes in the second series may have been an accidental infection.

The nature of this inactivation is not understood. An explanation of this process would probably at least suggest the

nature of the causal agency. The inactivation is not of the same nature as the killing of bacteria by the abiotic rays, since the time required is of an entirely different order. In the inactivation of the mosaic virus, exposures as long as 30 minutes are necessary, whereas, so far as known, all micro-organisms are killed by exposures which are measured in seconds rather than minutes.

The writer tried the influence of the rays from this same burner on *Bacillus prodigiosus*, with the idea of comparing the killing time here with the inactivation time in the case of the virus. Transfers of the organism were made, and when the cultures were 48 hours old a suspension was made in sterile distilled water. Plates were poured which contained 1 cc. each of a 1 : 10,000 and 1 : 100,000 dilution of the original suspension, in order to determine the density of the suspension. Counts of these plates showed that the original suspension contained approximately 12×10^5 organisms per cc.

The original suspension and a 1 : 1,000 dilution of that suspension were then treated by the same method as was the virus in the above experiment, 5 cc. of the suspension being removed with a sterile pipette and placed in sterile petri dishes which were kept covered except during the short periods of exposure. Duplicate exposures were made in each case, and two plates inoculated from each exposure. The plates each contained a 1-cc. sample from the 5-cc. portion exposed to the rays. The results obtained are tabulated in table II.

TABLE II

EFFECT OF ABIOTIC RAYS ON BACILLUS PRODIGIOSUS

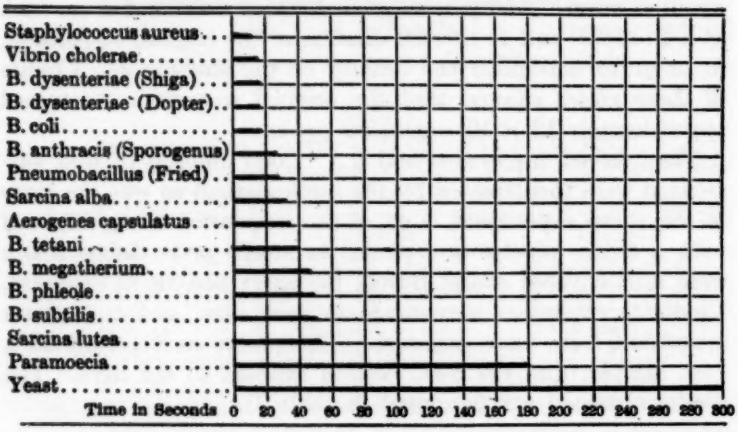
	Exposure			
	½ minute		1 minute	
	Series A	Series B	Series A	Series B
Orig. suspension	6 2	7 0	5 2	2 1
1 : 1,000 dilution	0 1	0 0	0 0	0 0

These results show that a suspension of *Bacillus prodigiosus*

of 12×10^6 per cc. can be practically killed by an exposure to the shorter ultra-violet rays given by the Burdick lamp when operated at 70 volts and 8 amperes. The killing time, which is 30 seconds in the case of *Bacillus prodigiosus*, therefore, is certainly not of the same order as the time of exposure required for inactivation of the virus. That this short killing time is a common characteristic of many organisms is shown in fig. 57 of Ellis and Wells ('25) which is included here as table III.

TABLE III

TIME IN SECONDS REQUIRED TO DESTROY VARIOUS ORGANISMS AT A DISTANCE OF 200 MM. FROM A QUARTZ MERCURY LAMP OPERATING AT 66 VOLTS AND 3.5 AMPERES



It is of interest to note from the table just referred to that the spores are not much more resistant to the abiotic effect of the rays than is the vegetative growth. According to Ellis and Wells von Recklinghausen has shown that while spores are 20 times more resistant than the vegetative forms to the action of chemical germicides, they are only 3 times more resistant to the ultra-violet rays than the vegetative forms.

These experiments show, then, that the abiotic rays can inactivate the virus if the latter is exposed to them for a sufficient length of time, but this time factor is many times greater than that which is necessary for the killing of the common micro-

organisms, either in spore or vegetative form. Hence, these results would seem to indicate that the virus is not an organism in nature. Whether the time factor is comparable to that which is necessary for the inactivation of enzymes has not yet been determined, and such determinations will give further indication as to the nature of the virus. The inactivation may simply have been due to a precipitation of certain proteins, since with the longer exposures a certain turbidity was observed in the formerly clear solution, and it is known that the rays will decrease the stability of the solution of some of the proteins, particularly the albumens.

2. Petunia sp.—A study of living free-hand sections of leaf tissues of healthy petunia plants and those infected with mosaic revealed in the latter the vacuolate bodies and the clear plate-like crystals similar to those observed so frequently in tobacco as studied particularly by Goldstein ('24). Just as in tobacco, the hair cells and the epidermal cells offered unusually favorable material in which to study the bodies in the living condition.

Although several leaves were studied from each of numerous healthy plants, no such inclusions were found. The normal petunia hair cell, as shown in pl. 13, fig. 1, contained only the nucleus, the cytoplasmic threads, and occasional small plastids carried along in the streams. The nucleus was found either suspended in the vacuole of the cell by the cytoplasmic threads, or closely pressed against the edge of the cell.

In the leaves of plants infected with mosaic, the hair cells in the dark green areas were similar to the healthy cells, showing no unusual inclusions. In the chlorotic areas, however, there were universally present both the vacuolate bodies and the plate-like crystals. Contrary to the distribution found by Goldstein in tobacco, there were never more than one or two of the vacuolate bodies present in a single cell at a given time. She illustrated as many as five in a single hair cell. The crystals, however, were present sometimes singly (pl. 13, fig. 2); sometimes as two or more separate and distinct crystals, each being carried about in the streams by itself; and sometimes in masses of numerous individuals lying adjacent to each other but not fused (pl. 13, fig. 19).

The cells were studied unstained as well as treated with various vital stains. The most successful vital stain employed was brilliant cresyl blue. The cells were mounted in water, and after they had been studied in the unstained condition a drop of a 1 : 10,000 solution of the stain was drawn in under the cover slip. The stain was taken up in from 15 minutes to 2 hours by the bodies, which could then be identified in the mesophyll, as well as in the epidermal hair cells.

The bodies exhibited different forms, varying from more or less definitely rounded, finely or coarsely granular structures, to irregular, vacuolate, amoeboid-like bodies. A limiting membrane was never observed except when the cells had been treated with 15 per cent alcohol and shrinkage had taken place, leaving visible the structure which was apparently a limiting membrane, as shown in pl. 13, fig. 9. The vacuoles varied in size, some bodies containing several large ones, and others numerous smaller ones giving them a porous or spongy appearance. They were not definitely associated with the nucleus as Künkel ('21) described them in corn, although at times they did appear adjacent to it. However, when the living cells were watched for a considerable length of time it was found that in those which were actively streaming the bodies were carried along in the streams. The nucleus frequently acted as an impediment in the course of the body, delaying its passage through the cell. If the cell had been observed only at that particular time the natural conclusion would have been that there was some association between the nucleus and the body, whereas a continued observation of the same cell showed that this was not the case. Never was one of these bodies seen in the process of division.

Single cells were watched for 2- and 3-hour periods of time, and the bodies and crystals observed as they were carried along in the protoplasmic streams. During such observations over long intervals of time, the bodies were seen to change in shape as well as to advance with the streams. The cell illustrated in pl. 13, fig. 2, was kept under observation for 2 hours, and during that time the body was perpetually changing form, as shown in the 40 camera-lucida sketches in text-fig. 1. The body apparently sent out short pseudopodium-like projections and these pro-

jections always appeared in the direction in which the body was being carried by the protoplasmic streaming. When meeting an obstacle such as the crystal, the nucleus, or some of the plastids, it would be stopped for a short time, then shape itself around the obstacle, and in a short time pass around it and continue to be carried in the stream. When reaching the end of the cell, the body could frequently be seen to flatten out in the stream against the cell wall and then again round up and continue its course back through the cell.

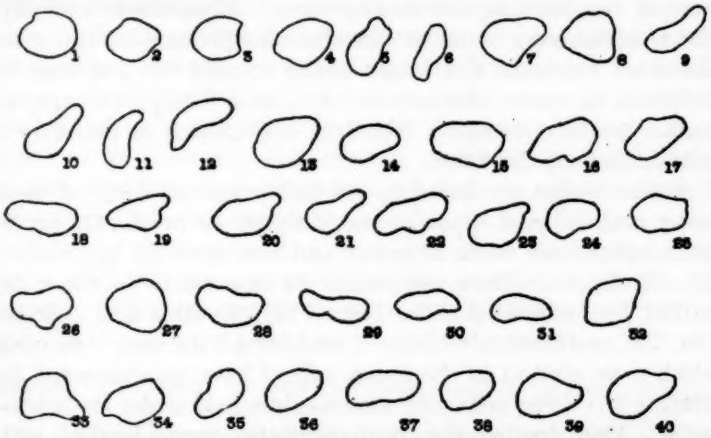


Fig. 1. Camera-lucida sketches showing changes in outline exhibited by the vacuolate body in the diseased hair cell of *Petunia* shown in pl. 13, fig. 2. These changes occurred during a two-hour period of observation while the body was being carried through the cell by the rapidly streaming protoplasm. × 1250.

At first, these movements were interpreted as being automotive, but further study seemed to show that they probably were not. If they were automotive, why did such movements occur only in those bodies in cells which were showing rapid streaming? They were never witnessed in cells unless there was a very pronounced cyclosis. Similar movements were observed by Goldstein ('24) in the bodies in the hair cells of tobacco and have been interpreted as sluggish amoeboid movements, but the author does not agree with her interpretation. If they were autonomous movements they should occur in the trichome cells independently of the streaming movements of the cyto-

plasm. Until further investigations have been conducted, such as attempting to remove the bodies from the cells by microdissection, it is impossible to state whether or not these bodies have automotive powers. At present, however, the author favors the view that the bodies are not the causal agency, but rather the result of a reaction between the cytoplasm and the virus. The apparent autonomous movements may have one of the 3 following explanations, or a combination of them. In the first place, these apparent changes may be due to the rolling over of the body in the moving stream of particles; secondly, the particles may be so mobile that the pressure of the cytoplasm as it pushes this larger object around the cell may be sufficient to cause changes in form, and finally, perhaps, to surface tension changes. The true explanation is probably a combination of the three.

Similar bodies are found in the fixed material, some of them being rounded and more or less definite, as in pl. 13, fig. 7, while others are more irregular and amoeboid in appearance (pl. 13, fig. 8). These are similar in appearance to those described by Iwanowski ('03), Palm ('22), Rawlins and Johnson ('24, '25), and Goldstein ('24). That they are the same structures which were studied in the living cells, I have demonstrated by treating the living cells with chrom-acetic acid under the microscope. Thus treated the fixed structures were identical with the bodies in the living cells.

The plate-like, highly refractive crystals were also studied. These, too, were observed to be transported in the cytoplasmic streams, but usually not as rapidly as the smaller bodies. As they were turned over by the pressure of the rapidly streaming cytoplasm, they showed various appearances, sometimes appearing long and narrow when viewed on edge; often appearing almost ribbon-like when viewed at an angle; and at other times as the flat plate-like structures shown in pl. 13, figs. 2 and 19. Their nature has not been determined, although the effect upon them of various solvents has been tried. They are definitely associated with the chlorotic areas of the leaves of plants infected with mosaic. They are not the result of a precipitation of products in the cell-sap, because they are definitely carried in the cyto-

plasmic streams. When studied in the living cells of tobacco by Dickson ('22) they were interpreted as being the product of chlorophyll degeneration combined with changed plastid protoplasm. His interpretation was based on the suggestion of Ewart's that CO_2 combined with chlorophyll in the presence of water to form xanthophyll and a colorless waxy substance. It seems improbable, however, that this is the explanation of the crystals obtained here, since in the epidermal and trichome cells in which they were studied the chloroplasts were few in number and very small in both the healthy and mosaic plants.

In the fixed material the crystals were not well preserved but were either striated in appearance or completely dissolved. These striated structures were observed by Iwanowski ('03) and interpreted as being plates of bacteria which were not visible except as the clear plate-like crystals in the living tobacco cells. As yet, no satisfactory explanation has been given of them, and there is none to be offered at present.

With the view of getting some indication of the nature of the bodies and the crystals in petunia, the solvent action of various substances, such as alcohol, formalin, KOH, HCl, HNO_3, and CH_3COOH were tried with the following results.

EFFECT OF ALCOHOL ON THE INCLUSIONS FOUND IN THE PETUNIA LEAVES INFECTED WITH MOSAIC

Free-hand sections of the chlorotic areas were made and studied in water mounts, thus insuring the presence of bodies and crystals. The sections were then placed in small vials containing approximately 1 cc. of the solvents, and examined at more or less frequent intervals during a 12-hour period. The solubility of the vacuolate bodies in the various dilutions was entirely different from that of the crystals, both products, however, being soluble in 95 per cent alcohol.

These results show that the bodies are soluble only in 95 per cent ethyl alcohol, whereas the crystals are attacked in as low as 10 per cent dilutions. Plate 13, figs. 9–12 inclusive, show bodies in cells which have been in 15, 30, 50 and 70 per cent alcohol for 12-hour periods, showing that they are not dissolved at those concentrations. When attacked by the alcohol, the crystals were seen to swell and eventually burst and become uniformly

TABLE IV

EFFECT OF DILUTION OF ALCOHOL ON SOLUBILITY OF INCLUSIONS

Solvent	Bodies	Crystals
C_2H_5OH 5%	Not affected	Not affected in 12 hrs.
C_2H_5OH 10%	Not affected	Dissolved after 6 hrs.
C_2H_5OH 15%	Slightly plasmolyzed	Dissolved immediately
C_2H_5OH 30%	Not affected	Dissolved
C_2H_5OH 50%	Not affected	Dissolved
C_2H_5OH 70%	Not affected	Dissolved
C_2H_5OH 95%	Dissolved	Dissolved

distributed throughout the cell. This swelling took place immediately in all dilutions down to 10 per cent, and in this latter case the crystals were not completely dissolved until they had been kept in the solution for 6 hours.

EFFECT OF FORMALIN ON THE INCLUSIONS FOUND IN PETUNIA LEAVES INFECTED WITH MOSAIC DISEASE

Both the bodies and the crystals were found to be fairly resistant to the action of formaldehyde in 4, 8, and 12 per cent concentrations, the following results being obtained.

TABLE V

SOLVENT ACTION OF VARYING CONCENTRATIONS OF FORMALIN ON INCLUSIONS

Solvent	Bodies	Crystals
Formalin 4%	Not affected	Not affected immediately, but some disintegration after 12 hrs.
Formalin 8%	Not affected	Show signs of disintegration
Formalin 12%	Some disintegration	Some disintegration

The disintegration accompanying the treatment of the cells with 12 per cent formalin was not specific for the inclusions alone, since there was also a general disintegration of the cell contents. Plate 13, fig. 19, shows a portion of a cell containing a mass of crystals and a single body which had been in 4 per cent formaldehyde for 1 hour. All of the inclusions were still perfectly normal in appearance, showing a resistance to the solvent action of formalin. After having been exposed to the same solvent for 12 hours, however, the crystals began to exhibit the effects of the solvent action, losing their angular corners, becoming rounded off, and occasionally showing signs of the dissolution of the crystal,

as in pl. 13, fig. 18. Results comparable to these were obtained at the end of 1 hour with 8 per cent formalin, as is shown in pl. 14, fig. 33. On the whole, however, one may conclude that the bodies and the crystals are relatively resistant to the solvent or disintegrating action of formaldehyde.

EFFECT OF ACIDS AND ALKALIS ON THE INCLUSIONS FOUND IN PETUNIA LEAVES INFECTED WITH MOSAIC DISEASE

To determine the effect of an alkali, KOH was used in concentrations ranging from .25 to 4 per cent. In all cases all of the inclusions were attacked immediately, the cell contents being rendered almost homogeneous, except in the case of the .25 per cent solution. In this dilute concentration dissolution was not immediate, but gradual. All of the inclusions, however, disappeared within 6 hours.

Diametrically opposite results were obtained regarding the solvent action of the mineral acids, HCl and HNO_3. With concentrations as high as 10 per cent neither the bodies nor the crystals were injured, although the cells were severely plasmolyzed, as is shown in pl. 14, fig. 34. The results with acetic acid, however, were entirely different, the bodies not being injured by any concentration up to 10 per cent, whereas the crystals were dissolved in 20 minutes in all concentrations above 1 per cent, and in that concentration they were also dissolved in 1½ hours. These results of the effect of acids have been tabulated in table VI.

TABLE VI

SOLVENT ACTION OF ACIDS ON INCLUSIONS

Solvent	Bodies	Crystals
HCl 5%	Not affected	Not affected
HCl 10%	Not affected	Not affected
HNO_3 10%	Not affected	Not affected
CH_3COOH 1%	Not affected	Dissolved in 1 hour
CH_3COOH 2%	Not affected	Dissolved in 20 minutes
CH_3COOH 4%	Not affected	Dissolved in 20 minutes
CH_3COOH 5%	Not affected	Dissolved in 20 minutes
CH_3COOH 10%	Not affected	Dissolved in 20 minutes

From these studies, it can be concluded that the vacuolate bodies resemble the crystals in being relatively resistant to formalin, HCl, and HNO_3, and soluble even in .25 per cent

KOH. They differ, however, in their solubilities in alcohol and acetic acid, the crystals being dissolved in 10–95 per cent concentrations of alcohol and in 1–10 per cent concentrations of acetic acid, whereas the bodies are soluble only in 95 per cent alcohol and never in acetic acid. It is hoped that further studies in this direction and in micro-dissection may reveal something more fundamental regarding the nature of both the bodies and the crystals, together with an explanation of their connection with the chlorotic areas in mosaic-infected petunia and tobacco plants.

3. Datura Stramonium Linn.—Healthy leaves of *Datura*, together with those of plants infected with mosaic, were studied in material which was fixed in chrom-acetic acid and stained with Haidenhain's iron alum haematoxylin and Orange G. Leaves of approximately the same age were taken from the healthy and the mosaic-infected plants from which the histological and cytological studies here reported were made. It was found in the histological studies that neither the green nor the chlorotic areas of the mosaic-infected plants are comparable to the normal, the green being thicker than the healthy but showing normal tissue differentiation and distribution of plastids; whereas the chlorotic areas are approximately equal in thickness to those of the healthy but have poorly differentiated palisade tissue and show a decrease in the number of plastids. These differences are brought out in text-fig. 2, and the measurements are given in table VII. The figures given in table VII are in each case the average of 50 measurements made on numerous sections of different pieces of fixed material.

TABLE VII

COMPARATIVE MEASUREMENTS OF TISSUES IN HEALTHY AND MOSAIC INFECTED DATURA

	Healthy	Green area of mosaic	Chlorotic area of mosaic
Thickness of leaf	107.2 μ	208.9 μ	124.7 μ
Thickness of upper epidermis	10. μ	15.8 μ	19.4 μ
Thickness of palisade	48.9 μ	96.0 μ	40.3 μ
Thickness of spongy mesophyll	40.3 μ	75.6 μ	48.1 μ
Thickness of lower epidermis	10. μ	19.4 μ	17.1 μ
No. of rows of palisade cells	1.	1.	1.
No. of rows of spongy mesophyll	4–5	5–6	3–4
Longest diameter of chloroplasts	4.4 μ	5. μ	4. μ

A comparison of the measurements for the dark green area with those of the chlorotic area shows that the thickness of the leaf in the dark green area is 1.68 times as great as that in the chlorotic region; the palisade tissue is 2.38 times as thick; and the spongy mesophyll only 1.56 times as deep. This shows that the difference between the dark green and the chlorotic areas of the mosaic-infected plants is greatest in the palisade layer.

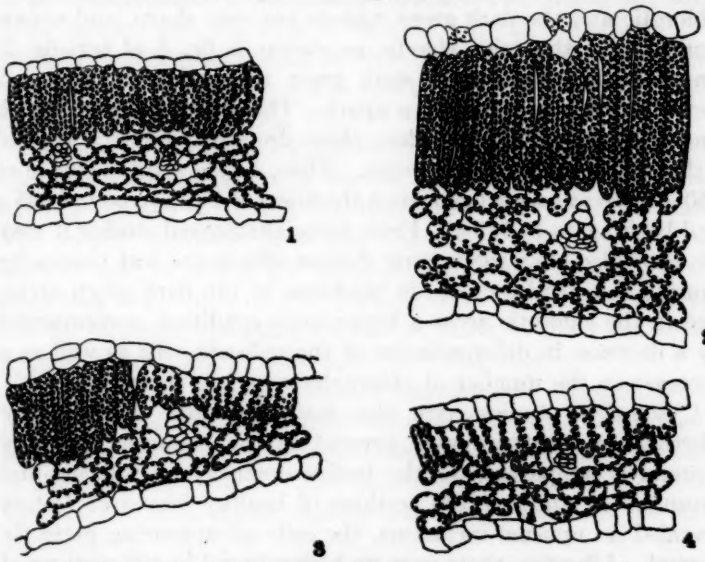


Fig. 2. *Datura Stramonium.* Semi-diagrammatic camera-lucida drawings of cross-sections of leaves, showing the effect of the mosaic on the tissues. × 300. 1, healthy leaf; 2, dark green area of a mosaic-infected leaf; 3, transitional area between the chlorotic and dark green areas of a mosaic-infected leaf; 4, chlorotic area of a mosaic-infected leaf.

This poor differentiation is shown more clearly in figs. 2, 3 and 4, of text-fig. 2.

On the other hand, a comparison of the measurements for the dark green areas of the mosaic-infected plants with those for the normal leaves shows that the increase in size of the dark green mosaic tissue over that of the healthy is approximately equal for all parts of the leaf. The thickness of the mosaic leaf in the dark green region is 1.96 times that of the healthy leaf; the upper epidermal cells are 1.94 times as deep; the palisade

layer is 1.96 times as high; the mesophyll layer is 1.88 times as thick; and the lower epidermis is 1.58 times as thick. Hence the dark green area, although exhibiting an enhanced thickness, shows no abnormal tissue differentiation. The plastid distribution, also, is similar in the dark green area of the mosaic-infected leaves and the healthy tissue.

In the diseased leaves, the transitional areas between the chlorotic and the dark green regions are very sharp, and apparently follow the veins closely, as shown in fig. 4 of text-fig. 2. In one case, there was a dark green area existing between 2 veins which were only 350 μ apart. The dark green area which measured 205 μ in the widest place dropped to 115 μ beyond either of the two limiting veins. Thus, in the short distance of 350 μ there was a change from a thickness of 115 μ to one of 205 μ and back again to 115 μ. From these anatomical studies it may be concluded that the mosaic disease affects the leaf tissues by causing a general increase in thickness in the dark green areas, and in the chlorotic areas a hypoplastic condition, accompanied by a decrease in differentiation of the palisade cells as well as a decrease in the number of chloroplasts and in their size.

Cytological studies were also made in order to determine whether or not there were present in the chlorotic cells any structures comparable to the bodies described in tobacco and petunia. A study of 100 sections of healthy leaves of *Datura* revealed no unusual inclusions, the cells all appearing perfectly normal. Likewise, there were no bodies found in 100 sections of the dark green areas of leaves of diseased plants. However, a study of the chlorotic areas revealed certain more or less irregular and indefinite structures, particularly in the upper epidermal cells.

These bodies resembled those described by Kunkel in corn much more nearly than they did those which are present in tobacco and petunia. They showed a strong affinity for Orange G, and at first sight gave the appearance of cytoplasm precipitated around the nucleus. Further study, however, demonstrated that they were a type of intracellular inclusion found only in the chlorotic areas of the mosaic-infected leaves. They were always adjacent to or surrounding the nucleus, showed

nothing of the nature of a limiting membrane, and were irregular in shape, size, and outline. Usually they were uniformly granular as in pl. 13, fig. 13, but occasionally they were more vacuolate as in pl. 13, fig. 15. In the vacuolate bodies, dark blue-staining granules could be observed as in fig. 15. Structures, such as the one illustrated in pl. 13, fig. 16, were apparently young stages in the development of the bodies, being small, taking the stain very lightly, and showing a tendency to be vacuolate. An unusual condition was found in which the nucleus was completely surrounded by the body, which last in turn was attached to the cytoplasm (see pl. 13, fig. 17).

At the time when the studies on the fixed material were made, no living diseased plants of *Datura* could be found, and consequently studies on living material have not yet been made. The author, however, feels that the bodies described here are definite entities which are associated with the chlorotic areas, since they were found only in those regions. When fresh material can be obtained observations will be made in order to see the appearance of these structures in the living cells.

4. Phytolacca decandra Linn.—Healthy and mosaic-infected poke were studied both in fixed material and in the living condition, the anatomical investigations being conducted as were those in the leaves of *Datura*. The following figures in each case are the average of 50 measurements which were made on the fixed material.

TABLE VIII

COMPARATIVE MEASUREMENTS OF TISSUES IN HEALTHY AND MOSAIC-INFECTED POKEWEED

	Healthy	Green area of mosaic	Chlorotic area of mosaic
Thickness of leaf	148 μ	191.5 μ	117.5 μ
Thickness of upper epidermis	15 μ	16. μ	15.5 μ
Thickness of palisade	57 μ	64.4 μ	27.3 μ
Thickness of spongy mesophyll	68.5 μ	100.2 μ	64.2 μ
Thickness of lower epidermis	7.5 μ	10.9 μ	11.0 μ
No. of rows of palisade cells	1.	1.	1.
No. of rows of mesophyll cells	±4.	±4.	4.
Longest diameter of chloroplasts	5. μ	5. μ	5. μ

Here, as in *Datura*, it can be seen that the mosaic disease modifies the entire leaf, the dark green area being thicker than

normal and the chlorotic region being reduced, particularly in the palisade layer. In this case also the healthy leaf is thinner than the dark green mosaic area but thicker than the chlorotic region. The thinness of the chlorotic sections is due chiefly to a lack of differentiation of the palisade layer, the cells of this last being 2.36 times thicker in the dark green than in the chlorotic regions whereas the mesophyll is only 1.71 times as thick. The chloroplasts are greatly reduced in number, particularly in the palisade layers, but their average size is not modified. As shown

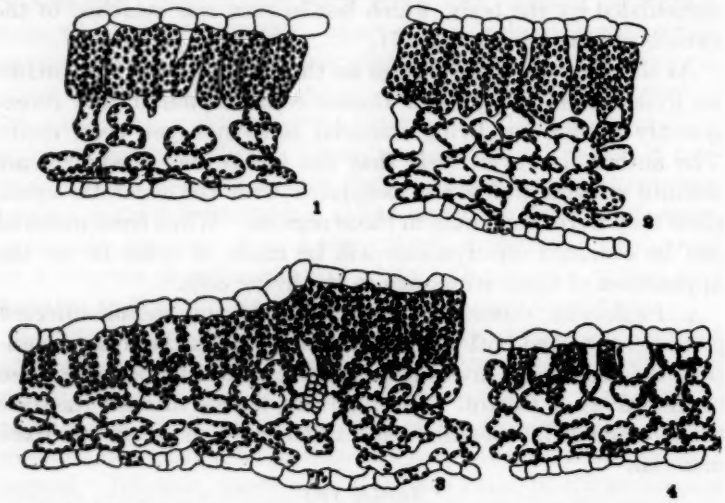


Fig. 3. *Phytolacca decandra*. Semi-diagrammatic camera-lucida drawings of cross-sections of leaves, showing the effect of the virus on the tissues. × 300. 1, healthy leaf; 2, dark green area of a mosaic-infected leaf; 3, transitional area between the chlorotic and dark green areas in a mosaic-infected leaf; 4, chlorotic area of a mosaic-infected leaf.

in fig. 3 of text-fig. 3, the transitional area is gradual and not sharp as it is in *Datura*, and the mottling may or may not follow the veins.

In addition to the anatomical study, cytological studies were made in order to locate any inclusions in the mosaic cells which were not in the healthy leaves. Very rarely bodies were found in the chlorotic areas, and although they were not of as common occurrence as in the other mosaics studied they were never

found in healthy leaves nor in the dark green areas of the diseased plants. Figs. 21–24 of pl. 14 illustrate the types of bodies found.

One type of structure found was that shown in the epidermal cell in pl. 14, fig. 21. Here the body was very definite in outline, stained orange-brown, and filled with numerous very small vacuoles, some of which contained dark blue-staining granules. There seemed to be no connection between it and the nucleus in this cell or in any other cell in which similar bodies were found. This, however, was not the typical kind of structure.

The more typical bodies were similar to the one in pl. 14, fig. 23. Here, the body took a faint blue-gray stain, contained several large vacuoles, and showed no limiting membrane. All such bodies appeared rounded rather than amoeboid in shape, and were but little denser than the surrounding cytoplasm, taking, however, a slightly different stain. In the other plants infected with mosaic disease, these vacuolate bodies showed a definite affinity for the Orange G, but in poke the blue-gray color indicated that there was some affinity for the haematoxylin. Such inclusions as these were more frequent than the others. In one case there appeared a very unusual modification of this type, as shown in pl. 14, fig. 22. Aside from its unusual shape, it was similar in all respects to the vacuolate bodies such as the one illustrated in pl. 14, fig. 23. Such bodies were found both in the epidermal and spongy mesophyll cells, but because of their poor staining reactions were sometimes difficult to study.

Figure 24, of pl. 14, shows a most unusual structure. It was very definite in outline, apparently being composed of 5 distinct component parts. It was definitely walled and took a differential stain, the periphery appearing orange and the central portion of each component part staining blue. This may have been an artifact, but it seemed too definite and it was thought to be of sufficient interest to be included here.

These structures were not abundantly distributed throughout the cells, but inasmuch as they were found only in the chlorotic areas, 100 sections of healthy leaves and a similar number of sections of dark green areas from mosaic-infected plants revealing nothing of the sort, it seemed of interest to include them here since they possibly accompany the chlorotic areas in mosaic-infected poke leaves.

5. Aquilegia.—Since 1919 Duggar has been observing a mosaic infection on plants of *Aquilegia* at the Missouri Botanical Garden. The diseased plants develop the typical mosaic symptoms including leaf mottling (see pl. 16, fig. 46), dwarfing of the plant, and decreased flower production. His inoculation experiments have indicated that it can be more or less readily transmitted. The infection was found on *Aquilegia caerulea* James, and since none of the healthy material of this species could be found, leaves of the hybrid *A. canadensis* Linn. × *A. californica* Gray were used for comparison with the plants infected with mosaic. Anatomical studies were made as in *Datura* and in pokeweed, and the measurements tabulated in a similar manner, as follows:

TABLE IX

COMPARATIVE MEASUREMENTS OF TISSUES IN HEALTHY AND MOSAIC-INFECTED AQUILEGIA

	Healthy	Green Area of mosaic	Chlorotic area of mosaic
Thickness of leaf	110 μ	150 μ	100 μ
Thickness of upper epidermis	15 μ	20 μ	20 μ
Thickness of palisade	42.5 μ	50 μ	29 μ
Thickness of mesophyll	38.5 μ	57.5 μ	32.5 μ
Thickness of lower epidermis	15 μ	20 μ	20 μ
No. of rows of palisade cells	2	2	1
No. of rows of mesophyll cells	±4	±4	±4

These measurements, together with the semi-diagrammatic drawings in text-fig. 4, show that the most striking difference between the chlorotic and the dark green areas is a loss of one of the palisade layers in the former; although there is also a decrease in the thickness of the mesophyll, that in the dark green area being 1.76 times as great as that in the chlorotic sections. Here, again, the healthy leaves are thinner than the dark green areas and thicker than the chlorotic regions. The chloroplasts show a gradual degeneration in the transitional areas between the dark green and chlorotic areas, as shown in fig. 3 of text-fig 4. This loss of an entire palisade layer in the chlorotic areas is similar to the condition described by Funaoka ('24) in some of the variegations, e. g., that in *Richardia Elliottiana*, and *Euphorbia marginata*, in which there is a loss of certain layers of tissue in the chlorotic areas. The chlorotic appearance is therefore due

to the absence of one of the palisade layers as well as to a decrease both in the number of plastids and the chlorophyll content of these.

Cytological studies were also made on the living and the fixed material of both healthy and mosaic-infected leaves, but, although 100 sections were studied of each, there were no inclusions of any

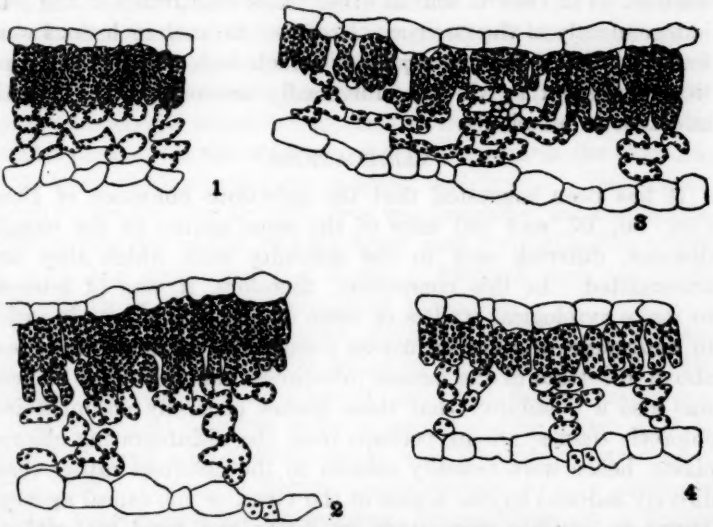


Fig. 4. *Aquilegia.* Semi-diagrammatic camera-lucida drawings of cross-sections of leaves, showing the effect of mottling on the tissue. × 300.
1, *Aquilegia californica* × *A. canadensis*, healthy leaf; 2, *A. caerulea*, dark green area of an infected leaf; 3, *A. caerulea*, transitional area between chlorotic and dark green areas of an infected leaf; 4, *A. caerulea*, chlorotic area of an infected leaf.

sort found in the chlorotic areas which did not also exist in the healthy and in the dark green portions.

From these studies on the tissues of healthy and mosaic-infected plants of *Datura*, pokeweed, and *Aquilegia*, it has been shown that the virus causes 2 changes in the leaves. First, it apparently stimulates certain areas to a general increase in thickness, and second in other sections of the leaves it causes a marked inhibition of growth, the reduction being chiefly in the palisade cells, and in the number and size of plastids, and the amount of chlorophyll which they contain. The transitional

areas may follow the veins and be fairly sharp, as in *Datura*, or they may not be necessarily associated with the vascular system, at which times they are very gradual, as in pokeweed. Associated with the chlorotic areas in tobacco, petunia, and *Datura*, possibly also in pokeweed, there were certain irregular vacuolate or granular bodies which at times were associated with the nucleus, as in *Datura*. and at other times distributed in the cells independently of the nucleus. However, no such inclusions were found in the tissues of *Aquilegia* which were studied, showing that perhaps they do not universally accompany the mosaic infection in all plants.

VARIEGATIONS

It has been suggested that the infectious chloroses of Baur ('04, '06, '07, and '08) were of the same nature as the mosaic diseases, differing only in the difficulty with which they are transmitted. In this connection, therefore, it was of interest to make cytological studies of some of his variegations in order to determine whether inclusions comparable to those described above in certain of the mosaic infections could be found. There was also a possibility that these bodies generally accompanied chlorotic tissues, arising perhaps from the disintegrating chloroplasts, hence were causally related to the chlorosis rather than directly induced by the action of the virus, or the causal agency. Therefore, studies were made on living and fixed material of various non-infectious variegations as well. A preliminary survey was made of many of those which were at the author's disposal, and from these the following were selected for more intensive study because they represent families in both the Monocotyledons and the Dicotyledons, because there are among them both infectious and non-infectious chloroses, and because they exhibit very different and distinct anatomical variations which, in some cases, are entirely different from those found in the mosaic plants infected with mosaic which have been studied.

1. Homalomena cordata Schott.—The variegation in this species consists of completely chlorotic round spots which, from microscopic observations, do not follow the veins, as shown in pl. 15, fig. 44. Free-hand sections were studied in the living condition, as well as sections of material which had been killed in chrom-

acetic acid, sectioned, and stained with Haidenhain's iron alum haematoxylin and Orange G.

Anatomical studies showed no difference in tissue differentiation between the green and the chlorotic areas. In neither region were the palisade cells developed, the leaf sections merely consisting of the upper epidermis, approximately 6 layers of spongy mesophyll, and the lower epidermis. The chief difference was the number and size of the chloroplasts; in the green areas, they were exceedingly large (averaging 8 μ in the longest diameter) and were more abundant, particularly in the upper layer of the spongy mesophyll which corresponds to the palisade layer in the ordinary leaf. On the contrary, the chloroplasts in the chlorotic areas were smaller and more sparsely distributed. The transitional region between the two areas was not sharp, the decrease in number and size of the plastids being very gradual. The nuclei in both the green and the chlorotic sections were strikingly large, both in the living and fixed materials.

Cytological studies, however, revealed no inclusions comparable in any way to those found in the plants infected with mosaic, although 100 sections of the fixed tissues and many living free-hand sections were examined. The living cells of both the green and chlorotic areas were frequently found filled with highly refractive bodies approximately 5 μ in diameter which were constantly in motion. Nevertheless, it is highly improbable that these bodies have any causal relation to the variegation, since they were found equally abundant in both the green and the chlorotic area. They were not observed in the fixed material.

2. Ficus Parcellii Veitch.—The variegation here is a mosaic-like mottling as shown in pl. 15, fig. 39. The different areas were very distinct, angular in appearance, and exhibited various distinct shades of green as well as pure white. Both living and fixed material were studied. Tissue taken from a nearly green leaf, such as that in pl. 15, fig. 45, was found to consist of the following layers of tissue,—upper epidermis, 2 layers of palisade cells, 4 layers of spongy mesophyll, and the lower epidermis, all of which contained chloroplasts. A study of the variegated leaves showed that the different colors of green had distinct

anatomical explanations, being due to a difference in distribution of the chloroplasts in the various layers. Transitional areas were always very sharp, one cell containing numerous chloroplasts, while the adjacent one might be completely devoid of them. It was found that there were 4 possible variants, as follows:

a. First palisade layer.
b. Second palisade layer.
c. First two rows of spongy mesophyll cells.
d. Lower two rows of spongy mesophyll cells.

Any one or any combination of these variants might be chlorophyll-free, while the remainder would be filled with chloroplasts. Some of the combinations which were observed are the following:

1. a, chlorophyll-free; b, c, and d filled with plastids.
2. b, chlorophyll-free; a, c, and d filled with plastids.
3. c, chlorophyll-free; a, b, and d filled with plastids.
4. b and d, chlorophyll-free; a and c filled with plastids.
5. d, chlorophyll-free; a, b, and c filled with plastids.
6. a, b, and d, chlorophyll-free; c filled with plastids.
7. a and b chlorophyll-free; c and d filled with plastids.

There was therefore no tissue differentiation between the green and the chlorotic areas, the difference in color being entirely due to an absence of plastids in one or more layers of tissue.

Cytological studies of both living and fixed cells revealed no inclusions comparable to those associated with the mosaic diseases.

3. Bougainvillea glabra Choisy.—The variegation in this species consists of a marginal chlorosis which may at times affect the entire leaf, as shown in the 3 leaves of pl. 16, fig. 48. The green portions of the variegated leaves, however, are not as dark a green as are the normal leaves, such as those shown in pl. 16, fig. 54. Microscopic studies showed that this had an easily interpreted anatomical explanation, which was similar to that given for the various shades of green found in *Ficus Parcellii*. The normal green leaf consists of an upper epidermis, one layer of palisade cells, 5 layers of mesophyll, and the lower epidermis, with chloroplasts distributed throughout the palisade as well as the spongy mesophyll cells. In the variegated leaves,

however, chloroplasts were never present in the palisade cells, being distributed only in the upper two layers of spongy mesophyll or through the entire mesophyll other than the palisade. The chlorotic areas lacked all chlorophyll. Here, again, the transition between the green and chlorotic areas was sharp, one cell containing normal plastids and the cell adjacent lacking them completely. Such transitions usually occurred near the veins. There was no difference in tissue differentiation between the two areas, the leaves being of uniform thickness.

Cytological studies on both living and fixed material gave no evidence of any intracellular inclusions which might be interpreted as the causal agency of the chlorosis.

4. Pittosporum Tobira Ait.—The variegated leaves are a duller green than the normal leaves and have a completely chlorotic margin of varying depth, as shown in pl. 15, fig. 40, which may be compared with the green leaf in pl. 15, fig. 41. Here the normal green leaves consist of a thick upper epidermis, 2 or 3 layers of palisade tissue, 8 or 9 layers of spongy mesophyll, and a lower epidermis. The chloroplasts are distributed throughout the palisade and the spongy mesophyll, being more numerous in the former. In the green areas of the variegated leaves the distribution of the plastids is different, there being 2 layers of chlorophyll-free cells immediately below the upper epidermis and another 2 rows of similar cells immediately above the lower epidermis. Accordingly, there may be said to be a chlorotic mantle surrounding the green tissue, thus accounting for the duller green color of the leaves. Funaoka ('24) has termed this type of chlorosis "periclinal variegation" and has described it in variegations of *Pelargonium zonale*, *Glechoma hederacea*, *Acer Negundo*, etc.

The variegated leaves show a white margin which is sharply set off from the dull green tissue. A microscopic study of this white area showed a notable decrease in thickness of the leaf, both the palisade layers and the spongy mesophyll being reduced. Particularly were the intracellular spaces reduced. In such areas the chlorophyll was completely lacking.

Cytological studies were made on both fixed and living material, and in neither the green nor the chlorotic areas could any unusual

intracellular inclusions be observed in the 100 sections examined.

5. Nerium Oleander Linn.—Here, as in *Pittosporum Tobira*, the variegation consists of a chlorotic margin of varying depths, as shown in pl. 15, fig. 35, which is to be compared with the totally green leaves as shown in pl. 15, fig. 36. Microscopic studies show that the normal green leaf and the green portions of the variegated leaves are similar, except that in the entirely green leaf there are more chloroplasts in the lower part of the spongy mesophyll than there are in the variegated leaves. The green tissue consists in the order mentioned of an upper epidermis, 2 rows of chlorophyll-free cells, 2 rows of palisade cells, 6 rows of spongy mesophyll, 2 rows of chlorophyll-free cells, and a lower epidermis. The chlorotic areas show the same tissue differentiation, but the chloroplasts are replaced by leucoplasts throughout the spongy mesophyll and the palisade cells. The boundaries between the green and the chlorotic areas are very sharp, one cell showing the normal chloroplasts and the one adjacent exhibiting only leucoplasts. These transitions are always coincident with the veins. Occasionally, definite areas are observed which are lighter green than the normal, and this was found to be due to the fact that such areas lack chloroplasts in the upper palisade layer or in the spongy mesophyll cells.

Living and fixed tissues were studied cytologically but no unusual inclusions were found in either the green or the chlorotic areas.

6. Coleus Blumei Benth.—It was considered that perhaps the variety "Mrs. Kirkpatrick" of *Coleus*, fig. 37, pl. 15, was closely related to the mosaic infections, since it showed the pronounced crinkling of the leaves which is so characteristic of the mosaic symptoms. Sections of the leaves were studied in both the fixed and the living condition, but no intracellular inclusions other than degenerated chloroplasts could be found. In the fixed material the chloroplasts occasionally assumed appearances comparable to the vacuolate bodies in the tobacco and petunia mosaics, but they did not give the proper staining reactions, becoming more blue than orange. In the living cells they were not at all comparable to the vacuolate bodies in tobacco and would never be confused with them. Clear, highly refractive

bodies were seen in the living cells and were in constant motion as though exhibiting Brownian movement. They were found, however, in both the green and the chlorotic areas and could not be interpreted as a causal factor in the variegation.

Anatomical studies showed a condition different from any which have been described in this paper. The transition between the green and the chlorotic areas was so gradual as to be scarcely perceptible. The green area possessed two rows of palisade cells which gradually became shorter and thicker until they could no longer be distinguished from the spongy mesophyll. Therefore, in the chlorotic areas there was no differentiation between the palisade and the spongy mesophyll.

7. *Evonymus japonica* Linn.—There were two different variegations of this species at the author's disposal, both of which were studied by Baur. The variety "*medio-picta*" is shown in fig. 49 of pl. 16, as contrasted with the normal green in fig. 50 and the other variegation, "*argenteo-variegata*," in fig. 51. Anatomical studies revealed similar tissue differentiation in the 2 variegated varieties, so it will be necessary to include only a discussion of one of them, var. "*medio-picta.*"

In this variegation the difference in tissue differentiation is found to be quite similar to that described for the *Datura* plants infected with mosaic except that here there are 3 layers of palisade cells. Measurements were made and tabulated as in the study of the plants infected with mosaic.

TABLE X

COMPARATIVE MEASUREMENTS IN GREEN AND VARIEGATED EVONYMUS LEAVES

	Normal green	Variegated green	Variegated chlorotic
Thickness of leaf	215 μ	420 μ	320 μ
Thickness of upper epidermis	20 μ	25 μ	20 μ
Thickness of palisade	150 μ	175 μ	100 μ
Thickness of spongy mesophyll	125 μ	200 μ	185 μ
Thickness of lower epidermis	20 μ	20 μ	15 μ

These data show that the green leaves are much thinner than either the chlorotic or green portions of the variegated leaves, and that the difference lies chiefly in the palisade layer, which

is 3.5 times greater in the green areas of the variegated leaves than in the green leaves. In the chlorotic areas the palisade layers are greatly reduced and contain relatively few chloroplasts, the ones that are present showing signs of disintegration. The transitional area between two regions usually covers about 5 or 6 cells.

Cytological studies were made on both the living and the fixed material of both types of variegations and of the normal green plant. The chlorotic areas of both vars. "*medio-picta*" and "*argenteo-variegata*" showed vacuolate bodies comparable to those found in tobacco and petunia mosaics, except that they occurred only in mesophyll and never in epidermal cells. Although at least 100 sections of the normal green tissue were studied, no such inclusions were found in these at any time.

The bodies were very similar in appearance to those in tobacco infected with mosaic. They showed a strong affinity for Orange G, contained several large or numerous small vacuoles, could be found adjacent to, or independent of, the nucleus, and occasionally, in the fixed material, exhibited numerous dark blue-staining granules (see pl. 14, figs. 25–32). No indication of a limiting membrane could at any time be observed. In the living cells the bodies appeared very similar to those in the fixed material, except that granules were never observed in any of the vacuoles.

Therefore, although these vacuolate bodies were not found in the non-infectious variegations studied, they were observed in the variegated varieties of *Evonymus japonica*. This is one of the species in which Baur ('08) found infectious chloroses. This would, then, appear to be evidence in favor of the view that these cell inclusions are associated directly with the virus rather than with the chlorosis which results from the presence of the virus, since they have been found in connection with only the one type of chlorosis—the infectious type. However, the author favors the view that they are not the causal agency itself but rather the product of a reaction between the virus and the cytoplasm of the cells.

Summary

1. Epidermal and hair cells of leaves of tobacco plants infected with the mosaic disease were examined in living and fixed tissues. The following observations of Goldstein were confirmed: the vacuolate bodies were not associated with the nuclei but were carried through the cells in the protoplasmic streams, the plate-like crystals were independent of the nuclei, and they lost their typical structure when placed in chrom-acetic acid. Contrary to her observations, however, the writer failed to observe any autonomous movements in the vacuolate bodies, and only once could the appearance of a limiting membrane be identified.

2. The observations of Rawlins and Johnson with reference to the fact that the inclusions occurred more frequently in greenhouse plants than in those grown out of doors were confirmed.

3. Treatment of mosaic-infected tobacco plants with the longer or biological ultra-violet rays for 18 days caused a dwarfing of the plants and a masking of the symptoms. Cytological studies of the rayed plants showed that the inclusions were present as in the controls, and these observations led to the conclusions that the absence of bodies in plants grown out of doors is not associated with the ultra-violet rays which they receive from the sun's spectrum.

4. The filtered mosaic tobacco juice was inactivated by an exposure of 30 minutes to the abiotic rays, whereas, under similar treatment, a suspension of *B. prodigiosus* was killed in 30 seconds. This is considered as evidence against the theory that the causal agency is an organism.

5. Living epidermal and hair cells of *Petunia* presented excellent material in which to study the intracellular inclusions. In cells showing rapid streaming, the vacuolate bodies exhibited 2 different movements: a migration through the cell, due to their being carried in the streams; and a change in form. The latter movement was explained as resulting from a combination of the effect of the force exerted on the mobile body by the streaming protoplasm and the apparent changes in form due to its turning over in the streams.

6. Regarding the reactions to solvents, the vacuolate bodies

and the plate-like crystals were alike in their relatively high resistance to the action of formalin, HCl, and HNO_3, and in the solubility in KOH. They differed, however, in the fact that the crystals were soluble in 10–95 per cent alcohol and 1–10 per cent acetic acid, whereas the vacuolate bodies were soluble only in 95 per cent alcohol and were not touched by acetic acid.

7. Anatomical studies of mosaic-infected leaves of *Datura*, pokeweed, and *Aquilegia*, showed that the virus enhanced the development of some areas and inhibited it in others, neither area, therefore, being normal. The reduction of tissue in the chlorotic regions was localized particularly in the palisade layers, and the chlorosis was accompanied by a decrease in size and number of chloroplasts.

8. Cytological studies of *Datura* revealed in the chlorotic areas irregular, granular, and vacuolate bodies in association with the nuclei, comparable to those described in corn by Kunkel. They were not found in the dark green areas of the diseased leaves or in the healthy tissues.

9. Cytological studies of pokeweed revealed intracellular vacuolate and sometimes granular bodies only occasionally present in the chlorotic areas.

10. No inclusions, not also present in the healthy cells, were found in the chlorotic areas of the diseased *Aquilegia*.

11. Anatomical studies were made on seven different variegations, and the variations in the difference between the chlorotic and green areas in the various types of chloroses were observed.

12. No inclusions were found associated with the chlorotic areas in *Homalomena cordata* Schott, *Ficus Parcellii* Veitch., *Bougainvillea glabra* Choisy var. *variegata*, *Pittosporum Tobira*, Ait. var. "*variegatum*," *Nerium Oleander* Linn., and *Coleus Blumei* Benth. var. "Mrs. Kirkpatrick."

13. Cytological studies revealed the presence of vacuolate bodies in the mesophyll cells of the chlorotic areas of *Evonymus japonica* vars. "*argenteo-variegata*" and "*medio-picta*." *Evonymus japonica* is one of the species in which Baur found infectious chloroses.

14. The vacuolate bodies, therefore, have been observed, as yet, only associated with the infectious chloroses.

15. These observations seem to justify the conclusion that the vacuolate and granular bodies discussed in this paper are associated directly with the causal agency rather than with the chlorosis which results from the presence of the virus in the plant. The author favors the view, however, that they do not represent the causal agency, but are rather the product of a reaction between it and the cytoplasm of the cells.

Acknowledgments

The writer wishes to express her sincere appreciation to Dr. B. M. Duggar for suggesting this problem and for his many kind and helpful criticisms throughout the work; to Dr. F. H. Ewerhardt, of Barnes Hospital, for the use of his mercury vapor lamps in the early part of the work; and to Dr. G. T. Moore for the privileges and facilities of the Missouri Botanical Garden.

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Explanation of Plate

PLATE 13

Fig. 1. *Petunia.* Living hair cell from leaf of healthy plant, showing only a nucleus and small plastids, and no inclusions. × 1000.

Fig. 2. *Petunia.* Living hair cell from the chlorotic area of a mosaic-infected leaf, showing, in addition to the nucleus and plastids, a typical vacuolate body near the plate-like crystal. × 1000.

Figs. 3–6. *Petunia.* Vacuolate bodies as seen in the living hair cells and epidermal cells. × 1000.

Fig. 7. *Petunia.* Rounded vacuolate body adjacent to the nucleus in an upper epidermal cell of the chlorotic area of a mosaic-infected leaf. The tissue was fixed in chrom-acetic acid, and stained with Haidenhain's haematoxylin and counterstained with Orange G. No formed structures or limiting membrane were present. × 2000.

Explanation of Plate (*Continued*)

Fig. 8. *Petunia.* Irregular amoeboid-like vacuolate body in a mesophyll cell immediately above the lower epidermis. Fixed and stained as in fig. 7. × 2000.

Fig. 9. *Petunia.* Vacuolate body as seen in a hair cell which had been kept in 15 per cent alcohol for a 12-hour period. The resulting shrinkage left visible a distinct limiting membrane. × 1000.

Fig. 10. *Petunia.* Nucleus and adjacent body of a cell which had been held in 30 per cent alcohol for 12 hours, showing that this concentration of alcohol neither dissolved nor materially modified these vacuolate bodies. × 1000.

Fig. 11. *Petunia.* Vacuolate body in a hair cell which had been kept in 50 per cent alcohol for 12 hours, showing that there were no injurious effects. × 1000.

Fig. 12. *Petunia.* Vacuolate body in a hair cell which had been placed in 70 per cent alcohol for 12 hours, showing no solution nor disintegration even at this high concentration. × 1000.

Fig. 13. *Datura Stramonium.* Upper epidermal cell in chlorotic area of a mosaic-infected leaf, showing the indefinite granular body adjacent to the nucleus. Several dark-staining granules are present in it. The leaf was fixed in chrom-acetic acid and stained in Haidenhain's iron alum haematoxylin and counterstained with Orange G. × 2000.

Fig. 14. *Datura Stramonium.* Nucleus and adjacent body in an upper epidermal cell in the transitional area between the chlorotic and green area in fixed material of a mosaic leaf. The body shows some suggestion of a vacuolate structure, but no limiting membrane. × 2000.

Fig. 15. *Datura Stramonium.* Nucleus with adjacent body in an upper epidermal cell of the chlorotic area in a mosaic-infected leaf. The material was fixed and stained as in fig. 13. This is a rather typical appearance of the body, being filled with small vacuoles in which there were occasionally dark-staining granules. × 2000.

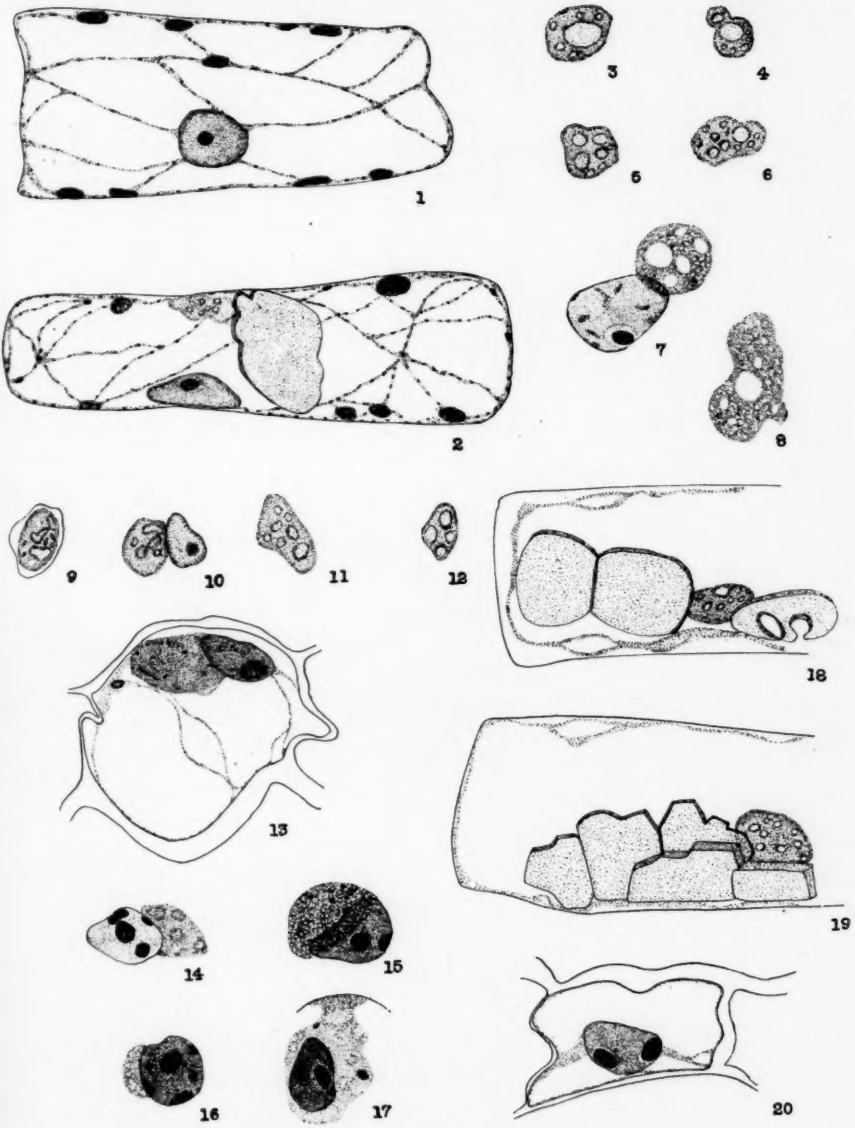
Fig. 16. *Datura Stramonium.* Apparently the young stage of a body. It was found in the upper epidermal cell in the chlorotic area of a mosaic-infected leaf which had been fixed and stained as in fig. 13. × 2000.

Fig. 17. *Datura Stramonium.* Unusual appearance of the body which completely surrounded the nucleus and was attached to the cytoplasm at the edge of the cell. Upper epidermal cell of chlorotic area. The body is less dense than usual and contains several dark-staining granules. × 2000.

Fig. 18. *Petunia.* Portion of hair cell from the chlorotic area of a mosaic-infected leaf, which had been placed in 4 per cent formaldehyde for a 12-hour period. The vacuolate body was still normal in appearance. The crystals had lost their sharp angular edges, and in the one on the right of the body disintegration and solution had set in. × 1000.

Fig. 19. *Petunia.* Portion of a hair cell from the chlorotic area of a mosaic-infected leaf which had been kept in 4 per cent formaldehyde for 1 hour. Both the vacuolate body and the plate-like crystals were normal in appearance. × 1000.

Fig. 20. *Datura Stramonium.* Healthy epidermal cell fixed in chrom-acetic acid and stained with Haidenhain's iron alum haematoxylin and Orange G. No inclusions were present. × 2000.



SMITH—MOSAIC DISEASES AND LEAF VARIEGATIONS

Explanation of Plate

PLATE 14

Fig. 21. *Phytolacca decandra.* Upper epidermal cell of the chlorotic area of a mosaic-infected leaf, showing the very regular rounded body containing numerous small vacuoles, some of which surround very small dark-staining granules. The leaf was fixed in chrom-acetic acid and stained with Haidenhain's iron alum haematoxylin and Orange G. × 1500.

Fig 22. *Phytolacca decandra.* Cell immediately above the lower epidermis in the chlorotic area of a mosaic-infected leaf, fixed and stained as in fig. 21. This most unusual body was vacuolate, took a light gray-blue stain, was only slightly denser than the cytoplasm, and apparently was not associated with the nucleus. × 1500.

Fig. 23. *Phytolacca decandra.* Cell just above the lower epidermis in the chlorotic area of a mosaic-infected leaf, fixed and stained as in fig. 21. The cell showed the typical appearance of the bodies, the latter being a clear blue-gray and containing several large vacuoles. Several chloroplasts were also present in the cell. × 1500.

Fig. 24. *Phytolacca decandra.* Cell in second row above the lower epidermis in the chlorotic area of a mosaic-infected leaf. The very definitely walled structure in the center of the cell took a deep orange stain in the periphery which surrounded and gradually changed into the dark blue-staining centers. In addition, the cell contained 9 chloroplasts and a nucleus. × 1500.

Fig. 25. *Evonymus japonica.* var. "*medio-picta.*" Mesophyll cell in the chlorotic area of the variegated leaf. The body contained a large vacuole, numerous small ones, and several dark-staining granules. It apparently was not associated with the nucleus which was in the lower part of the cell among several plastids. × 1500.

Fig. 26. *Evonymus japonica* var. "*medio-picta.*" Nucleus and adjacent body which were found in a cell next to the one in fig. 25. The body contained a single large vacuole with several small ones. The material was fixed in chrom-acetic acid and stained with Haidenhain's iron alum haematoxylin and Orange G. × 1500.

Fig. 27. *Evonymus japonica* var. "*argenteo-variegata.*" Mesophyll cell in the chlorotic area of the variegated leaf, containing only the nucleus and a vacuolate body. There were no dark-staining granules in the body, and only 2 large vacuoles but many small ones. The material had been fixed and stained by the usual method. × 1500.

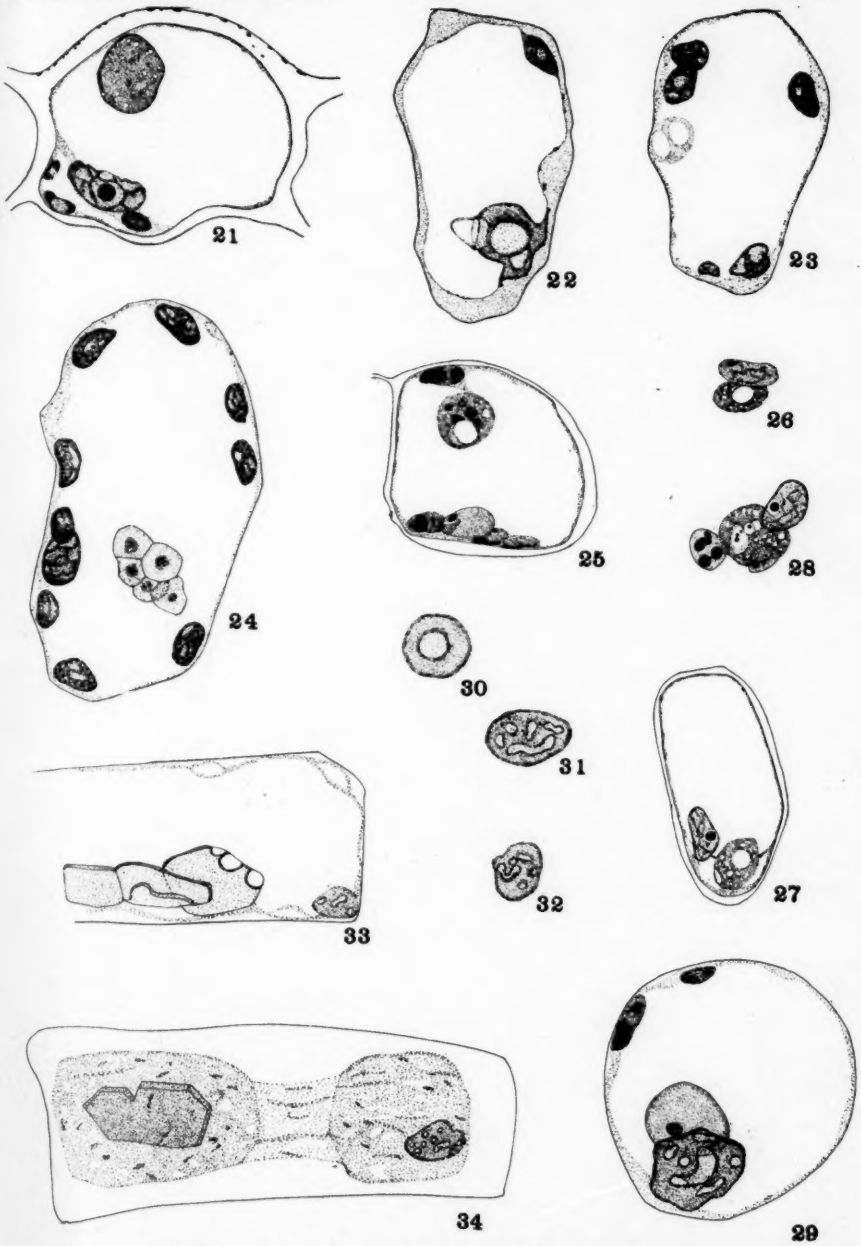
Fig. 28. *Evonymus japonica* var. "*argenteo-variegata.*" Vacuolate body in a mesophyll cell of the chlorotic area of a variegated leaf. It was surrounded by the nucleus and 2 disintegrating plastids. It contained two large vacuoles with dark-staining granules, and numerous small ones. The material was fixed and stained as in the previous figures. × 1500.

Fig. 29. *Evonymus japonica* var. "*medio-picta.*" Living mesophyll cell showing the nucleus with the vacuolate body partially superimposed. Two small chloroplasts were present in the cytoplasm. × 1500.

Figs. 30, 31, and 32. *Evonymus japonica* var. "*medio-picta.*" Vacuolate bodies as seen in the living mesophyll cells in the chlorotic areas of the variegated leaves. × 1500.

Fig. 33. *Petunia.* Portion of a hair cell from the chlorotic area of a mosaic-infected leaf which has been placed in 8 per cent formalin for 1 hour, showing the normal appearance of the vacuolate body but the beginning of disintegration of the crystals. × 750.

Fig. 34. *Petunia.* Hair cell from the chlorotic area of a mosaic-infected leaf which had been kept in 10 per cent HCl for 6 hours, showing that, although there had been very severe plasmolysis, neither the crystals nor the body were injured. × 750.



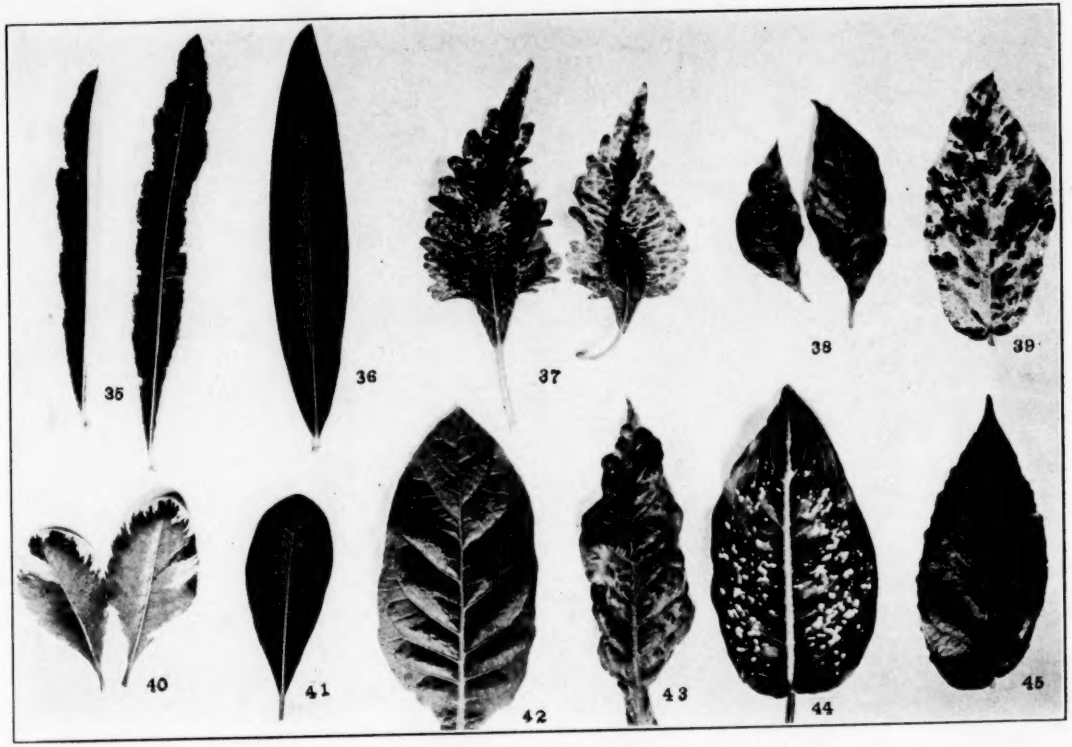
SMITH—MOSAIC DISEASES AND LEAF VARIEGATIONS

Explanation of Plate

PLATE 15

(Leaves one-half natural size)

Fig. 35. Leaves of the variegated variety of *Nerium Oleander* Linn.
Fig. 36. Normal green leaf of *Nerium Oleander* Linn.
Fig. 37. Leaves of *Coleus Blumei* Benth. var. "Mrs. Kirkpatrick."
Fig. 38. Mosaic-infected poke leaves.
Fig. 39. Variegated leaf of *Ficus Parcellii* Veitch.
Fig. 40. Leaves of *Pittosporum Tobira* Ait. var. *variegatum*.
Fig. 41. Normal green leaf of *Pittosporum Tobira* Ait.
Fig. 42. Healthy tobacco leaf.
Fig. 43. Mosaic-infected tobacco leaf.
Fig. 44. Leaf of *Homalomena cordata*.
Fig. 45. Nearly entirely green leaf of *Ficus Parcellii* Veitch.



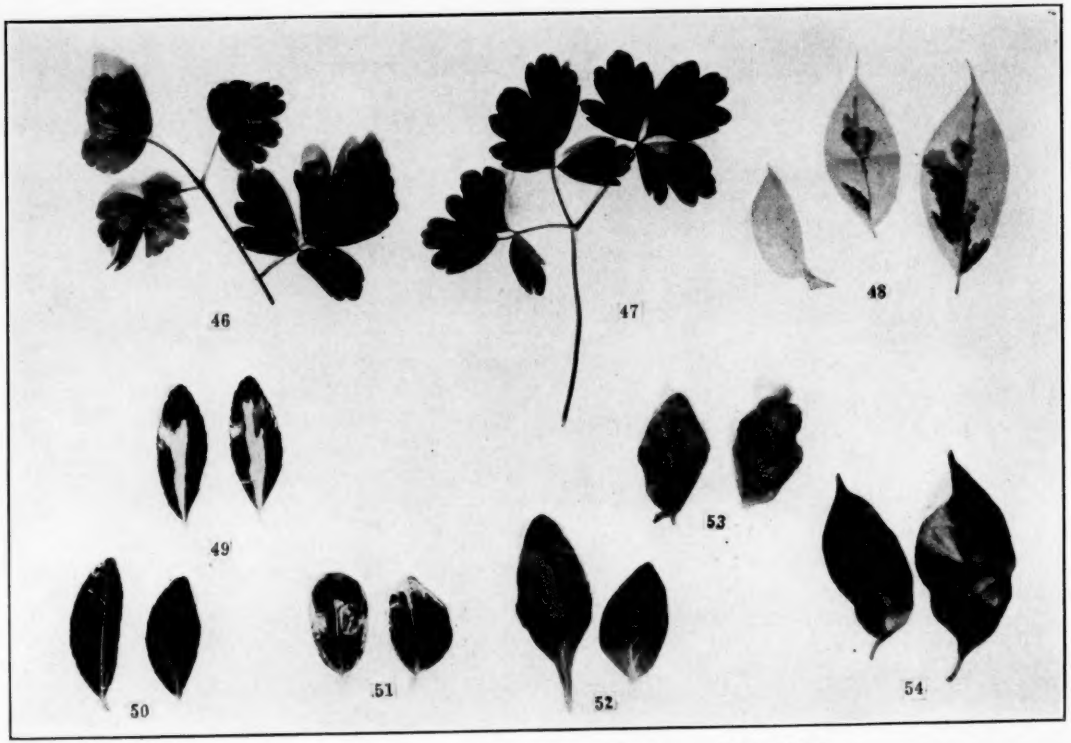
SMITH—MOSAIC DISEASES AND LEAF VARIEGATIONS

Explanation of Plate

PLATE 16

(Leaves two-thirds natural size)

Fig. 46. Leaves of mosaic-infected *Aquilegia caerulea* James.
Fig. 47 Leaves of healthy *Aquilegia.*
Fig. 48. Leaves of *Bougainvillea glabra* Choisy var. *variegata.*
Fig. 49. Leaves of *Evonymus japonica* var. *"medio-picta."*
Fig. 50. Normal green leaves of *Evonymus japonica* Linn.
Fig. 51. Leaves of *Evonymus japonica* var. *"argenteo-variegata."*
Fig. 52. Healthy *Petunia* leaves.
Fig. 53. Mosaic-infected *Petunia* leaves.
Fig. 54. Normal green leaves of *Bougainvillea glabra* Choisy.



SMITH—MOSAIC DISEASES AND LEAF VARIEGATIONS

GENERAL INDEX TO VOLUME XIII

New scientific names of plants and the final members of new combinations are printed in **bold face** type; synonyms and page numbers having reference to figures and plates, in *italics;* and previously published scientific names and all other matter, in ordinary type.

D

E

F

Q

R

S

T

U

V

X

Z